



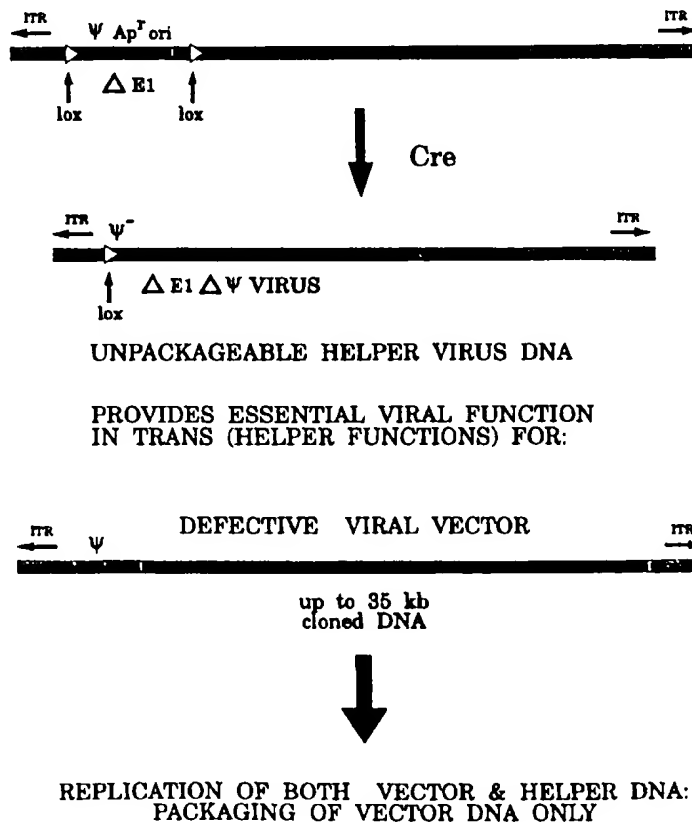
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification <sup>6</sup> : C12N 15/86</p>	<p>A1</p>	<p>(11) International Publication Number: WO 96/40955</p>	
		<p>(43) International Publication Date: 19 December 1996 (19.12.96)</p>	
<p>(21) International Application Number: PCT/CA96/00375</p>		<p>(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p>	
<p>(22) International Filing Date: 7 June 1996 (07.06.96)</p>		<p>Published With international search report.</p>	
<p>(30) Priority Data: 08/473,168 7 June 1995 (07.06.95) US</p>			
<p>(60) Parent Application or Grant (63) Related by Continuation US 08/173,168 (CIP) Filed on 7 June 1995 (07.06.95)</p>			
<p>(71)(72) Applicants and Inventors: GRAHAM, Frank, L. [CA/CA]; 34 Amelia Street, Hamilton, Ontario L8P 2C4 (CA). ANTON, Martina [DE/CA]; 1001 Main Street W., Hamilton, Ontario L8S 1A9 (CA). RUDNICKI, Michael, A. [CA/CA]; 14 Sherwood Rise, Dundas, Ontario L9H 4E8 (CA).</p>			
<p>(74) Agents: MORROW, Joy, D. et al.; Smart &amp; Biggar, 900-55 Metcalfe Street, P.O. Box 2999, Station D, Ottawa, Ontario K1P 5Y6 (CA).</p>			

**(54) Title:** ADENOVIRUS VECTORS FOR GENE THERAPY

**(57) Abstract**

This invention provides a method for making adenovirus cloning vectors which contain a recombinase target site that is useful for the insertion of selected foreign proteins.



*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

## ADENOVIRUS VECTORS FOR GENE THERAPY

This application is a continuation-in-part of Application Serial No. 08/250,885 filed on May 31, 1994 which is itself a continuation-in-part of Application  
5 Serial No. 08/080,727 filed June 24, 1993 from which priority is also claimed. Both of these applications are hereby incorporated by reference.

## FIELD OF THE INVENTION

The present invention relates to adenovirus  
10 vectors that have increased utility for gene transfer into mammalian cells. The vector systems described have increased capacity for insertion of foreign DNA and improved safety.

## BACKGROUND OF THE INVENTION

15 Parent Application Serial No. 08/250,885, filed on May 31, 1994, and its parent Application Serial No. 08/080,727 disclose and claim a genus of adenovirus (Ad)-derived cell expression vectors having excellent potential as live recombinant vaccines and as  
20 transducing vectors for gene therapy. In the human Ad genome, early region 1 (E1), E3, and a site upstream of E4 have been utilized as sites for introducing foreign DNA sequences to generate adenovirus recombinants. In the absence of compensating deletions in E1 or E3 a  
25 maximum of about 2 kb can be inserted into the Ad genome to generate viable virus progeny. The E1 region is not required for viral replication in complementing 293 cells and up to 3.2 kb can be deleted in this region to generate conditional helper independent vectors with a  
30 capacity of 5.0-5.2 kb. In the E3 region, which is not required for viral replication in cultured cells, deletions of various sizes have been utilized to generate nonconditional helper independent vectors with a capacity of up to 4.5-4.7 kb.

The maximum capacity for inserts of foreign DNA in currently available helper independent Ad vectors such as those described in the parent application is approximately 8 kb. This limited capacity arises from the use of Ad vectors which have deletions of E1 and E3 sequences and from the fact that most other regions of the viral genome must be retained in order that the viral vector may be propagated without the need for a helper virus.

Besides this limited capacity for insert DNA, previous vectors retain most of the viral genome, expression of viral genes in transduced cells or in inoculated animals, including humans, can result in toxic or other untoward effects. In addition, previous viral vectors can recombine with Ad sequences present in cells used for propagation of the vectors or with Ad sequences that may be present in inoculated animals. Therefore, it is the objective of this invention to provide Ad cloning vectors from which all or most viral genes have been removed and which will have increased safety and capacity for larger insertions compared to currently available vectors.

#### SUMMARY OF THE INVENTION

It is the goal of this invention to provide a simple and useful system by which high capacity Ad5 cloning vectors may be developed. As demonstrated in concurrently filed application serial No. 08/486,549 (entitled "Adenoviruses for Control of Gene Expression") provision of Cre recombinase in Ad infected cells can catalyze excision or rearrangement of viral DNA sequences that contain the target sites (loxP) for Cre mediated site specific recombination. In the present invention, use is made of this knowledge to construct Ad5 genomes in which the viral DNA packaging signals can be excised from the viral genome by action of Cre. Said excision of said packaging signal results in a viral DNA

that is unable to package into virion particles. Such a viral DNA, though unable to package into virions, may encode viral functions that provide complementing functions for replication of a second, viral "vector",  
5 that lacks substantial portions of the viral genome so that in coinfecting cells though both helper and vector DNAs may replicate, only the vector DNA can be packaged into virions.

One embodiment of the present invention  
10 provides a bacterial plasmid comprising a circularized modified human adenovirus type 5 (Ad5) genome that contains sequences that can be recognized and acted upon by a site specific recombinase known as Cre. Said  
bacterial plasmid is able to generate infectious Ad5  
15 carrying the modified sequences including the sequences that can be recognized by Cre. The structure of the modified sequences in the bacterial plasmid and in viruses generated from said plasmid is such that  
20 recombination catalyzed by Cre will result in excision of sequences, known as the packaging signal, near the left end of the Ad5 genome, that are required for packaging of Ad5 DNA into infectious virion particles. Optionally, certain regions of the plasmid and resulting  
25 viruses may be deleted, such as sequences from E1 or E3 that can be omitted from the viral genome without preventing the viral genome from replicating in such cells as may be permissive for replication of said viral genome in the form of infectious virus.

A second embodiment of the invention provides  
30 a bacterial plasmid comprising approximately 340 base pairs from the left end of the Ad5 genome, including the left end terminal repeat sequences of said genome and the packaging signal sequences thereof and the right  
terminal repeat sequences of the Ad5 genome. The left  
35 end of the left terminal repeat sequence is joined in "head to tail" configuration with the right end of the

right terminal repeat. Between approximately nucleotide 340 near the left end of the genome and approximately nucleotide 35,800 near the right end of the genome, are substituted restriction enzyme sites suitable for  
5 insertion of foreign DNA sequences.

A third embodiment of the invention provides a mammalian cell line, such as a human cell line, that provides the Cre recombinase enzyme. Alternatively, Cre may be provided by an Ad5 derived vector that expresses  
10 the Cre protein in suitable cells.

Other embodiments of the present invention include Ad genome constructs, known as "vectors", containing substantial deletions of viral DNA sequences that are substituted with large insertions of foreign  
15 DNA 20-35kb in length. Such genomes are unable to replicate as viruses in the absence of viral products provided by a second virus, hereafter called a "helper" virus.

One specific embodiment of the invention is a  
20 helper virus that can be designed, propagated, and used in such a way that when employed to support replication of a second virus, the vector, from which substantial portions of the viral genome have been deleted and substituted with foreign DNA, said "helper" virus DNA is  
25 unable to be packaged into infectious virions.

#### BRIEF DESCRIPTION OF FIGURES

**Fig. 1** is a diagrammatic representation of Cre mediated excision of DNA from a viral vector in which the packaging signal is flanked by *lox P* sites.

30 **Fig. 2** is a diagrammatic representation of a method to generate helper dependent viral vectors using Cre mediated excision of the packaging signal to prevent packaging of the helper virus DNA.

**Fig. 3** is a diagrammatic representation of a plasmid  
35 derived from pBHG10 into which *lox P* sequences have been introduced at positions flanking the packaging signal.

**Fig. 4** is a diagrammatic representation of a plasmid derived from pBHG10 from which most of the viral DNA has been deleted save for the left and right ITRs and the packaging signal.

- 5 **Fig. 5** is a diagrammatic representation of a means to obtain coreplicating helper and helper dependent viruses by cotransfection of 293Cre cells.

**Fig. 6** is a diagrammatic representation of the construction of pLC2 and pLC3 plasmids.

- 10 **Fig. 7** is a diagrammatic summary of the transformation, selection and screening protocol for Cre expressing 293 and 293N3S cell lines.

- Fig. 8** is a Western blot analysis of G418 resistant clones screened for expression of Cre with Cre specific  
15 rabbit antiserum.

**Fig. 9** is a diagrammatic representation of luciferase expression assays on parental cell lines.

**Fig. 10A** is a photomicrograph showing the results of luciferase expression assays on transformed cell lines.

- 20 **Fig. 10B** is a diagrammatic representation of luciferase expression assays on transformed cell lines.

**Fig. 11A** is a diagrammatic representation of the construction of AdLC8 helper virus.

- Fig. 11B** is a photomicrograph showing the ethidium  
25 bromide staining results demonstrating the efficiency of excision of packaging signals from AdLC8.

**Fig. 11C** is a photomicrograph showing Southern blot analysis results demonstrating the kinetics of Cre-mediated recombination.

- 30 **Fig. 12A** is a diagrammatic representation of the pRP1001 transfection of 293Cre1 cells and of the AdLC3 infection of the transfected cells 18 hours later.

- Fig. 12B** is a graph showing the titer increase resulting from the serial passage of AdRP1001 through AdLC8-  
35 infected 293 Cre1 cells.

Fig. 13A is a diagrammatic representation of the amplification of the AdRP1001 vector to high titers with AdLC8cluc.

Fig. 13B is a graph showing the titer increase resulting from the serial passage of AdRP1001 through AdLC8cluc-infected 234Crel cells.

Fig. 14 is a graph showing the relationship between luciferase activity and contaminating helper virus.

#### DETAILED DESCRIPTION OF THE INVENTION

Any publications referenced herein are hereby incorporated by reference in this application in order to more fully describe the state of the art to which the present invention pertains.

It is important to an understanding of the present invention to note that all technical and scientific terms used herein, unless otherwise defined, are intended to have the same meaning as commonly understood by one of ordinary skill in the art. The techniques employed herein are also those that are known to one of ordinary skill in the art, unless stated otherwise.

Reference to particular buffers, media, reagents, cells, culture conditions and the like, or to some subclass of same, is not intended to be limiting, but should be read to include all such related materials that one of ordinary skill in the art would recognize as being of interest or value in the particular context in which that discussion is presented. For example, it is often possible to substitute one buffer system or culture medium for another, such that a different but known way is used to achieve the same goals as those to which the use of a suggested method, material or composition is directed.

The terms used herein are not intended to be limiting of the invention. For example, the term "gene" includes cDNAs, RNA, or other polynucleotides that



encode gene products. "Foreign gene" denotes a gene that has been obtained from an organism or cell type other than the organism or cell type in which it is expressed; it also refers to a gene from the same  
5 organism that has been translocated from its normal situs in the genome. In using the terms "nucleic acid", "RNA", "DNA", etc., we do not mean to limit the chemical structures that can be used in particular steps. For example, it is well known to those skilled in the art  
10 that RNA can generally be substituted for DNA, and as such, the use of the term "DNA" should be read to include this substitution. In addition, it is known that a variety of nucleic acid analogues and derivatives can be made and will hybridize to one another and to DNA  
15 and RNA, and the use of such analogues and derivatives is also within the scope of the present invention. "Expression" of a gene or nucleic acid encompasses not only cellular gene expression, but also the transcription and translation of nucleic acid(s) in  
20 cloning systems and in any other context. The term "recombinase" encompasses enzymes that induce, mediate or facilitate recombination, and other nucleic acid modifying enzymes that cause, mediate or facilitate the rearrangement of a nucleic acid sequence, or the  
25 excision or insertion of a first nucleic acid sequence from or into a second nucleic acid sequence. The "target site" of a recombinase is the nucleic acid sequence or region that is recognized (e.g., specifically binds to) and/or acted upon (excised, cut  
30 or induced to recombine) by the recombinase. The term "gene product" refers primarily to proteins and polypeptides encoded by a nucleic acid, but further encompasses nucleic acids encoded by other nucleic acids (e.g., non-coding and regulatory RNAs such as tRNA,  
35 sNRPs). The term "regulation of expression" refers to events or molecules that increase or decrease the

synthesis, degradation, availability or activity of a given gene product.

The present invention is also not limited to the use of the cell types and cell lines used herein.

5 Cells from different tissues (breast epithelium, colon, lymphocytes, etc.) or different species (human, mouse, etc.) are also useful in the present invention.

It is important in this invention to detect the generation and expression of recombinant nucleic  
10 acids and their encoded gene products. The detection methods used herein include, for example, cloning and sequencing, ligation of oligonucleotides, use of the polymerase chain reaction and variations thereof (e.g., a PCR that uses 7-deaza GTP), use of single nucleotide  
15 primer-guided extension assays, hybridization techniques using target-specific oligonucleotides that can be shown to preferentially bind to complementary sequences under given stringency conditions, and sandwich hybridization methods.

20 Sequencing may be carried out with commercially available automated sequencers utilizing labelled primers or terminators, or using sequencing gel-based methods. Sequence analysis is also carried out by methods based on ligation of oligonucleotide  
25 sequences which anneal immediately adjacent to each other on a target DNA or RNA molecule (Wu and Wallace, Genomics 4: 560-569 (1989); Landren et al., Science 241: 1077-1080 (1988); Nickerson et al., Proc. Natl. Acad. Sci. 87: 8923-8927 (1990); Barany, F., Proc. Natl. Acad. Sci. 88: 189-193 (1991)). Ligase-mediated covalent  
30 attachment occurs only when the oligonucleotides are correctly base-paired. The Ligase Chain Reaction (LCR), which utilizes the thermostable *Taq* ligase for target amplification, is particularly useful for interrogating  
35 late onset diabetes mutation loci. The elevated reaction temperatures permits the ligation reaction to

be conducted with high stringency (Barany, F., PCR Methods and Applications 1: 5-16 (1991)).

The hybridization reactions may be carried out in a filter-based format, in which the target nucleic acids are immobilized on nitrocellulose or nylon membranes and probed with oligonucleotide probes. Any of the known hybridization formats may be used, including Southern blots, slot blots, "reverse" dot blots, solution hybridization, solid support based sandwich hybridization, bead-based, silicon chip-based and microtiter well-based hybridization formats.

The detection oligonucleotide probes range in size between 10-1,000 bases. In order to obtain the required target discrimination using the detection oligonucleotide probes, the hybridization reactions are generally run between 20°-60° C, and most preferably between 30°-50° C. As known to those skilled in the art, optimal discrimination between perfect and mismatched duplexes is obtained by manipulating the temperature and/or salt concentrations or inclusion of formamide in the stringency washes.

The cloning and expression vectors described herein are introduced into cells or tissues by any one of a variety of known methods within the art. Such methods are described for example in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1992), which is hereby incorporated by references, and in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, MD (1989), which is also hereby incorporated by reference. The methods include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors.

The protein products of recombined and unrecombined coding sequences may be analyzed using

immune techniques. For example, a protein, or a fragment thereof is injected into a host animal along with an adjuvant so as to generate an immune response. Immunoglobulins which bind the recombinant fragment are harvested as an antiserum, and are optionally further purified by affinity chromatography or other means. Additionally, spleen cells may be harvested from an immunized mouse host and fused to myeloma cells to produce a bank of antibody-secreting hybridoma cells. The bank of hybridomas is screened for clones that secrete immunoglobulins which bind the recombinantly produced fragment. More specifically, immunoglobulins that selectively bind to the variant polypeptides but poorly or not at all to wild-type polypeptides are selected, either by pre-absorption with wild-type proteins or by screening of hybridoma cell lines for specific idiotypes that bind the variant, but not wild-type, polypeptides.

Nucleic acid sequences capable of ultimately expressing the desired variant polypeptides are formed from a variety of different polynucleotides (genomic or cDNA, RNA, synthetic oligonucleotides, etc.) as well as by a variety of different techniques.

The DNA sequences are expressed in hosts after the sequences have been operably linked to (*i.e.*, positioned to ensure the functioning of) an expression control sequence. These expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors contain selection markers (*e.g.*, markers based on tetracycline resistance or hygromycin resistance) to permit detection and/or selection of those cells transformed with the desired DNA sequences. Further details can be found in U.S. Patent No. 4,704,362.

Polynucleotides encoding a variant polypeptide include sequences that facilitate transcription (expression sequences) and translation of the coding sequences such that the encoded polypeptide product is produced. Construction of such polynucleotides is well known in the art. For example, such polynucleotides include a promoter, a transcription termination site (polyadenylation site in eukaryotic expression hosts), a ribosome binding site, and, optionally, an enhancer for use in eukaryotic expression hosts, and, optionally, sequences necessary for replication of a vector.

*E. coli* is one prokaryotic host useful particularly for cloning DNA sequences of the present invention. Other microbial hosts suitable for use include bacilli, such as *Bacillus subtilis*, and other enterobacteriaceae, such as *Salmonella*, *Serratia*, and various *Pseudomonas* species. Expression vectors are made in these prokaryotic hosts which will typically contain expression control sequences compatible with the host cell (e.g., an origin of replication). In addition, any number of a variety of well-known promoters are used, such as the lactose promoter system, a tryptophan (Trp) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. The promoters typically control expression, optionally with an operator sequence, and have ribosome binding site sequences, for example, for initiating and completing transcription and translation.

Other microbes, such as yeast, are used for expression. *Saccharomyces* is a suitable host, with suitable vectors having expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an origin of replication, termination sequences, etc. as desired.

In addition to microorganisms, mammalian tissue cell culture is used to express and produce the

polypeptides of the present invention. Eukaryotic cells are preferred, because a number of suitable host cell lines capable of secreting intact human proteins have been developed in the art, and include the CHO cell lines, various COS cell lines, HeLa cells, myeloma cell lines, Jurkat cells, and so forth. Expression vectors for these cells include expression control sequences, such as an origin of replication, a promoter, an enhancer, and necessary information processing sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from immunoglobulin genes, SV40, Adenovirus, Bovine Papilloma Virus, and so forth. The vectors containing the DNA segments of interest (e.g., polypeptides encoding a variant polypeptide) are transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation is useful for other cellular hosts.

The method lends itself readily to the formulation of test kits for use in diagnosis. Such a kit comprises a carrier compartmentalized to receive in close confinement one or more containers wherein a first container contains suitably labeled DNA probes. Other containers contain reagents useful in the localization of the labeled probes, such as enzyme substrates. Still other containers contain restriction enzymes, buffers etc., together with instructions for use.

The recombinant Ad vectors described herein are significantly different from previously described constructs. They combine the use of vectors having deletions of all or most of the viral genes with helper viruses that are designed so that, when used in

coinfections with vector viruses, said helper viruses are able to complement the growth of the vectors but are unable to package their viral DNA into infectious virions. Thus vector viruses can be prepared

5 substantially free of helper virus.

For viral DNA replication and packaging of viral DNA into virion particles, only three regions of the viral DNA are known to be required in *cis*. These are the left inverted terminal repeat, or ITR, (bp 1 to

10 approximately 103) the packaging signals (approximately 194 to 358 bp) (Hearing and Shenk, 1983, Cell 33: 695-703; Grable and Hearing 1992, J. Virol. 64: 2047-2056) and the right ITR. All other regions of the viral genome appear to be required only to produce viral products

15 that act in *trans* to allow viral replication and production of infectious viruses. Thus if all essential viral proteins and RNA could be provided by a helper virus, a vector could be designed and constructed that could have most of the viral DNA deleted save for those

20 sequences mentioned above that are required in *cis* for viral DNA replication and packaging. A problem with helper dependent vectors has been that preparations of such vectors are invariably contaminated with helper virus and it is technically very difficult to separate

25 the helper from the vector. In the main embodiments of the present invention the helper virus is designed to have two *lox P* sites near the left end of the genome, one inserted at approximately 189bp from the extreme left end of the viral DNA, and the second, in parallel

30 orientation with the first *lox P*, situated rightward of the packaging signals, ie rightward of bp 358 (diagrammed in Figure 1). This virus will be able to replicate in cells that are normally permissive for growth of Ad5. However, in cells that express the Cre recombinase, or in the presence of a second virus that

35 expresses Cre recombinase, excision of sequences between

the lox P sites of the helper virus DNA will remove the packaging signal, and the resulting viral DNA will fail to package into infectious viral particles. Therefore, in cells coinfectd with said helper and with a second  
5 virus, a vector from whose genome have been deleted all or most of the viral DNA sequences that are normally required for expression of viral products necessary in trans for viral replication, both vector and helper viral genomes will replicate but only the vector DNA  
10 that retains the packaging signal will be packaged into virions (Figure 2).

In one embodiment of the invention, the helper virus is derived from a plasmid similar to ones described in the parent application and as illustrated  
15 in Figures 3 and 4. In these examples, the Ad5 genome is present as a circular molecule containing a bacterial plasmid derived origin of DNA replication (designated "ori") and a bacterial antibiotic resistance coding sequence ("Ap<sup>r</sup>") conferring to bacteria carrying said  
20 molecule resistance to ampicillin. In this example in which said circular form of the Ad5 genome is designated pBG17, viral sequences from regions E1 and E3 have been deleted from the viral genome, but this example is not meant to be limiting since other deletions or no  
25 deletions may equally be engineered in the circularized molecule by methods described in the parent application. The molecule designated pBG17 contains Ad5 sequences from bp 19 (left genomic end) to bp 341 with an artificially engineered BamH 1 restriction site inserted  
30 between approximately bp 188 and 189 in the Ad 5 sequences which is between the "ITR" and the packaging signal, " $\psi$ ", and known not to interfere with viral replication (Bett, A. J., W. Haddara, L. Prevec, and F. L. Graham. 1994, Proc. Natl. Acad. Sci. USA 91: 8802-  
35 8806). Ad 5 sequences present in pBG17 then extend rightward of the packaging signal to approximately bp



341 at which position is located an Xba 1 restriction site followed by Ad 5 sequences from approximately bp 3534 to approximately bp 27864, then sequences comprising 1874 bp of DNA containing the pUC19 origin of replication and ampicillin resistance gene, and finally Ad5 bp 30996 to 35934 (right genomic end). By techniques that are readily employed by a person skilled in the art, lox P sites, which are well defined DNA sequences of about 34 bp, can be introduced into the Ad5 genome at the Bam HI and XbaI sites flanking " $\psi$ ". For example synthetic double stranded oligodeoxynucleotides can be readily designed and synthesized such that they contain the lox P sequence recognized by cre and are flanked by single stranded extensions that allow ligation into BamHI or XbaI cleaved DNA. Thus a person skilled in the art can readily obtain the plasmid designated pBG17Lox1 (Fig. 4) having a lox P site introduced into the Bam HI site at nt 188, and subsequently pBG17Lox2 (Fig. 3) having an additional lox P site introduced into the XbaI site of pBG17Lox1. The plasmid pBG17 can be used to generate infectious virus by transfection of 293 cells. Equally, the plasmids pBG17Lox1 or pBG17Lox2, will generate infectious virus (eg. AdBG17Lox2 illustrated in Figure 3) since insertions of up to 271 bp can be engineered between the ITR and the packaging signal without interfering with viral replication and packaging of viral DNA (Hearing et al., Journal of Virology Vol 61, p 2555, 1987). In the presence of Cre enzyme the sequences containing the packaging signal will be excised as a result of intramolecular recombination between the two lox P sites (Fig. 3, bottom) resulting in a viral genome that retains all the sequences necessary for replication but lacks the sequences needed for packaging of DNA into virions. Said genome may serve as a complementing viral genome to support the replication of a second virus, a

vector, that lacks all or most of the viral genes necessary for viral replication as diagrammed in Fig. 2. These examples are not meant to be limiting as it will be appreciated that modified viruses similar in DNA  
5 structure to that of Figure 3 can be generated by other means. For example a person skilled in the art could introduce lox P sites into other sites in the plasmids illustrated in Fig. 3 such as the Bst B1 or Pac I sites or into such other locations as might be desirable, or  
10 into such other plasmids containing Ad sequences, or other Ad viral genomes as might be desirable. Use of Cre recombinase in this and other examples is not meant to be limiting as a person skilled in the art will readily appreciate that other enzymes capable of catalyzing site  
15 specific recombination between DNA sequences recognized by said enzymes could equally be employed in place of the Cre recombinase. An example, not meant to be limiting, of such an enzyme that could be substituted for Cre is the "FLP" recombinase of yeast in combination  
20 with its target site (O'Gorman et al. Science 251, 1351, 1991).

Another embodiment of the invention provides human cells, such as 293 cells or other cells that may be deemed suitable in that they support the replication  
25 of the viral components of the invention, that express Cre recombinase and that can be transfected with the plasmids described in the previous examples to generate a helper virus from which the packaging signals have been removed through excision mediated by Cre. It will  
30 be appreciated by those skilled in the art that the requisite cell lines can be generated by transfecting 293 cells or other cells with a plasmid comprising the coding sequences for Cre under the control of suitable regulatory sequences including a promoter and  
35 polyadenylation signal and containing in addition a selectable gene encoding, for example, resistance to

G418 or histidinol. A person skilled in the art can readily obtain drug resistant cells that will express the Cre recombinase in addition to the drug resistance gene used for selection.

5 In another embodiment of the invention, a plasmid consisting of sequences comprising the left ITR, the packaging signal, and the right ITR, and optionally containing additional viral sequences can be readily obtained. An example, which is not meant to be limiting, is illustrated in Fig. 4. In this example, pBG17Lox1 DNA  
10 is digested with restriction enzymes XbaI and SmaI which cleave the viral DNA in pBG17Lox1 at sites shown, as well as at other sites in viral DNA. The fragment containing the junction of viral termini (indicated by  
15 head to head arrows in Fig. 4) and the lox P site and packaging signal, can be purified and inserted into the polycloning site of a suitable cloning plasmid such as pUC18 or pUC19 to generate the plasmid designated as pPAD1. This example is not meant to be limiting as a  
20 person skilled in the art could equally insert said fragment into such other cloning plasmids as might be suitable or desirable. In the example illustrated, pPAD1 can serve as a vector for insertion of foreign DNA up to approximately 30 kb in size at one of the remaining  
25 restriction enzyme cloning sites present at the junctions of pUC and Ad5 DNA, to generate a plasmid such as pADHDV1, in which the open segment of pADHDV1 represents foreign DNA of arbitrary origin and sequence composition. The plasmid pADHDV1 contains all the Ad5  
30 sequences needed in cis for viral DNA replication and packaging of viral DNA into virions. Provided that viral functions necessary in trans are supplied by a helper virus, therefore, pADHDV1 will have the potential to replicate as a helper dependent viral DNA molecule that  
35 will contain up to 30 kb of foreign DNA flanked by viral DNA sequences from the left and right ends of the viral

genome. It may be advantageous to include as part of the foreign DNA inserted in pADHDV1, a DNA sequence capable of providing expression of a readily detectable reporter gene in addition to other sequences, the reporter gene  
5 providing a simple means of identifying cells or groups of cells that are infected with the virus ADHDV1 derived from pADHDV1. As an example which is not meant to be limiting, a person skilled in the art could include in pADHDV1, sequences coding for bacterial  $\beta$ -galactosidase,  
10 expression of which is readily detectable by exposure of cells to X-gal. Furthermore, in the example shown in Figure 4, pPAD1 and pADHDV1 contain a single lox P site at Ad5 nt 189, that is at the same site as for one of the lox P insertions in pBG17Lox1&2. Although this  
15 example is not meant to be limiting, placement of a lox P site at this position in pPAD1, pADHDV1, and derivatives, may serve to reduce the efficiency of recombination between helper virus and vector during coreplication of the two viruses as illustrated in  
20 Figures 2 and 5.

In another embodiment of the invention, coreplication of helper virus comprising sequences derived from a plasmid such as pBG17Lox2 and a helper dependent virus comprising sequences derived from a  
25 plasmid such as pADHDV1 may be achieved by cotransfection of cells with said plasmids to generate replicating viral genomes. In the example illustrated in Figure 5, which is not meant to be limiting, AdBG17Lox2 will, in the presence of Cre recombinase, be converted  
30 to AdBG17 $\psi$ - by excision of the sequences bracketed by lox P sites. The virus AdBG17 $\psi$ - will, by virtue of the removal of the packaging signals, be unable to package its genome into virions but will be able to replicate its DNA and provide viral functions necessary in trans  
35 for viral replication and thereby provide complementing

functions for replication of the helper dependent virus, AdHDV1. Because AdHDV1 retains the packaging signals the DNA of this helper dependent virus will be packaged into virions. The helper dependent virus AdHDV1 may be recovered, and optionally purified and concentrated by isopycnic centrifugation in CsCl gradients to produce helper dependent virus preparations substantially free or totally free of contaminating helper virus.

In another embodiment of the invention, 293 cells or other human cells that do not express Cre may be transfected with a plasmid such as that designated as pBG17Lox2 in Figures 3 and 5 to produce a virus such as that designated as AdBG17Lox2. Said virus may replicate in said cells without undergoing excision of sequences bracketed by lox P and can therefore be readily propagated. Coinfection of 293Cre or equivalent cells with AdBG17Lox2 and AdHDV1 will lead to formation of AdBG17 $\psi$ - which will complement the growth of AdHDV1 resulting in coreplication of both viral genomes but packaging only of AdHDV1 DNA into viral particles.

**Example 1. Production and Characterization of Cell Lines Expressing Recombinase Cre.**

Plasmid pLC2 was created by cloning the 1.6 kb XbaI/SalI fragment of pSP13cre into the XbaI/SalI site of pCA14, placing the cre gene under the control of the HCMV promoter and adding an SV40 polyadenylation signal (pA) to the 3' end of the gene. As the XbaI/SalI segment of pSP13cre already contains an SV40 pA, this cloning step resulted in two polyadenylation signals 3' of Cre. The 2.2 kb BglII fragment of pLC2 was then cloned into the BamHI site of pPBdx4. The resulting plasmid pLC3, contains the Cre expression cassette plus the gene for neomycin resistance under the control of

the SV40 promoter. Fig. 6 outlines the construction of the pLC2 and pLC3 plasmids. The structures of plasmids pLC2 and pLC3 were confirmed through restriction analysis.

5                   293 and 293N3S cells were maintained in F11 medium supplemented with 100 U penicillin per ml, 100 µg of streptomycin per ml, 2.5 µg/ml fungizone, and 10% fetal bovine serum for maintenance of 5% horse serum for infection. The cells were transformed with pLC2 using  
10 the calcium phosphate technique (McKinnon, R. D., and F. R. Graham, Microinjection and Organelle Transplantation Techniques 199-214 (1986)). Cells were then subjected to selection with 400 µg/ml G418 in complete F11 starting 3 days after transformation. Four weeks after  
15 transformation, G418 resistant colonies were cloned and expanded. Fig. 7 summarizes the transformation, selection and screening protocol for Cre expressing cell lines. Cell culture media and reagents with the exception of fungizone, were purchased from Gibco BRL  
20 (Burlington, Ontario, Canada). Fungizone was purchased from Bristol-Myers Squibb Canada (Montreal, Quebec, Canada).

Clones were initially screened for expression of Cre by Western blot analysis with Cre specific rabbit  
25 antiserum. Of 77 G418 resistant clones isolated, 7 293 and 17 293N3S clones were found to express Cre protein of the correct size (38kD) at levels varying from line to line. Ten representative lines are shown in Fig. 8. Lines 293Cre1 to 6 (lanes 2-7) and 293N3SCre7 and 8  
30 (lanes 9-10) were found to express high levels of Cre protein (comparable to levels in AdCre 1 infected cells; AdCre1 contains the cre gene under the control of the HCMV promoter in an E1 deleted vector) whereas no Cre was detected in parental 293 and 293N3S cell lines  
35 (lanes 1 and 8), or in 293N3SCre9 and 10 (lanes 11-12). A small amount of Cre had been detected in the latter

two cell lines when these clones were first isolated, but expression was undetectable in subsequent assays.

Cell lines that scored positive for Cre expression by Western blot were next assayed for functional recombinase activity using Ad vectors expressing reporter genes under the control of a Cre sensitive molecular switch (Anton, M. and F. L. Graham, J. Virol. 69:4600-4606 (1995)). 293Cre and 293N3SCre cell lines were infected with the vectors in combination with Add170-3 or AdCre1 at an MOI of 10 PFU per cell for each vector, to compare the levels of activity of the endogenously expressed Cre recombinase with those produced by AdCre1.

Clones that tested positive for Cre expression by Western blot analysis were then screened for Cre recombinase activity using the Cre inducible viruses AdMA19, AdMA23, and AdMA35. 293Cre and 293N3SCre cell lines were infected with the vectors in combination with Add170-3 or AdCre1 at an MOI of 10 PFU per cell for each vector, to compare the levels of activity of the endogenously expressed Cre recombinase with those produced by AdCre1. A preliminary screening using AdMA23, identified 6 293 Cre cell lines and 4 293N3SCre lines that were able mediate specific Cre-lox recombination and induce expression of  $\beta$ -galactosidase activity from AdMA23 in the absence of any extraneous source of Cre recombinase to at least 50% the levels obtained following a mixed infection with AdMA23 and AdCre1.

Because of the greater sensitivity and range of luciferase versus  $\beta$ -galactosidase assays, more detailed characterization of the lines was carried out using vector, AdMA19, that expresses luciferase following Cre mediated excision of a "spacer" DNA sequence. Parental 293 and 293N3S cell lines served as negative controls for levels of luciferase expressed

from AdMA19 in the absence of Cre expression, and coinfections with AdMA19 and AdCre1 were used as positive controls since it had been demonstrated previously that such mixed infections resulted in efficient excision of the spacer between the HCMV promoter and luciferase cDNA and induced high levels of luciferase expression.

As in the  $\beta$ -galactosidase assays, Add170-3 was substituted for AdCre1 in functional assays for endogenous expression of Cre in transformed lines to maintain the total amount of infecting Ad5 at constant levels. The results of a typical analysis are shown in Fig. 9 for parental cells and the 10 transformed lines that had scored positive in the preliminary screening. After infection with AdMA19 and Add170-3 most of the transformed lines expressed luciferase at efficiencies comparable to levels seen in parental 293 or 293N3S cells doubly infected with AdMA19 and AdCre1 whereas mock infected lines, or parental lines doubly infected with AdMA19 and Add170-3 did not express luciferase above background levels. Furthermore, infection of lines 293Cre1-6 and 293N3SCre7-8 with AdMA19 and AdCre1 did not significantly increase the amounts of luciferase made over those induced by infection with AdMA19 and Add170-3, in agreement with the results of the Western blot analysis showing levels of endogenously expressed Cre in lines 108 were similar to levels produced in AdCre1 infected 293 cells. Lines 293N3SCre9 and 10, on the other hand, initially expressed only low levels of luciferase following infection with AdMA19 and Add170-3, and significantly higher levels (5 fold and 12 fold greater, respectively) when Add170-3 was substituted with AdCre1 (Fig. 9).

Presently, these lines are no longer capable of inducing Cre responsive vectors without AdCre1 infection. This is consistent with current Western blot



analysis (Fig. 8, lanes 11-12), which indicated a lack of Cre expression in these lines. It is likely that the lines 293N3SCre9 and 293N3SCre10 initially contained functioning Cre expression cassettes that were lost on continued passaging of the cells.

**Example 2. Stability of Cre Cell Lines.**

The stability of the Cre cell lines after multiple passages in media with or without G418 was determined. DNA from 293Cre1, 293Cre3, and 293Cre4 and 293N3SCre8 was isolated after 9 or more passages with or without G418 selection. For digestion, EcoR1, which does not cut within pLC3 was chosen in order to detect changes in the copy number of the cre gene easily. A digoxigenin-dUTP labelled probe was made using the 1.6 kb XbaI/SalI fragment of pSP13cre, and hybridized to EcoR1 restricted chromosomal DNA. The probe hybridized with an approximately 9.5 kb fragment of 293Cre1, and to a 15 kb fragment of 293N3SCre8, indicating that cre sequences were present in genomic DNA of these lines (Fig. 10A). The probe also detected a very faint band at approximately 18 kb in 293Cre1, which may represent either an insert of a subfragment of the cre gene or incompletely digested DNA. Southern blot analysis of HindIII restricted DNA, which cuts once in pLC3 at a site outside cre and flanking regulatory sequences, indicated that cre sequences were present at a single copy in 293Cre1, and approximately 3 copies in 293N3SCre8. Both the 9.5 kb EcoRI fragment of 293Cre1 and the 15 kb fragment of 293N3SCre8 remained unchanged in cells maintained in the absence of G418 for 9 passages, with DNA for 293Cre1 retaining cre sequences after 18 passages. The cre probe detected intense bands at over 23 kb and 5 kb in 293Cre3, and intense 12.5, 9.8, and 6.6 kb bands from DNA of 293Cre4 cells maintained under G318 selection, indicating that cre sequences were present in these lines at 2 and 3 copies

respectively (Fig. 10A). These results were confirmed by hybridization of the probe to HindIII restricted DNA. The fainter bands detected at 20, 18, and 15 kb in 293Cre3 cells may represent partially restricted DNA. When cells were passaged 9 times in the absence of G418, the 6 kb fragment of 293Cre3 and the 6.6 kb fragment of 293Cre4 were no longer detected, indicating that the corresponding cre inserts in these cell lines had been lost. The remaining cre specific fragments within 293Cre3 and 294Cre4 appeared to be stable. Assays of plating efficiencies of lines 293Cre 1, 3, and 4, and 293N3SCre8 all indicated that G418 resistance was a stable phenotype for all lines, regardless of whether the cells were maintained in selective or nonselective media.

Several 293Cre lines grown with or without G418 selection were also tested for functional Cre activity. Cells were infected with a new  $\beta$ -galactosidase expression vector, AdMA35, in combination with Add170-3 or AdCre1, at an MOI of 10 pfu per cell for each virus as described previously. Cells were harvested 24 hours post-infection and  $\beta$ -galactosidase levels were determined. The results shown in Fig. 10B indicate that ability to induce expression of  $\beta$ -galactosidase from AdMA35 was generally unaltered after 10 or more passages in the absence of G418 selection. Functional activity of Cre in lines 293Cre1 and 293N3SCre8 was consistent with the stable presence of cre in Southern blot analysis. Thus cre sequences are stably integrated in these lines. The loss of one of two cre specific inserts in line 293Cre3 as detected by Southern blot (Fig. 10A), may coincide with a slight decrease in Cre function of these cells, as determined by assays with Cre inducible reporter genes (Fig. 10B) whereas the loss of one cre specific band in 293Cre4 appeared to have had no effect upon levels of functional

Cre. It is possible that sufficient Cre is expressed from the stable inserts to bind all *loxP* sites introduced into this cell line. Alternatively, the unstable insert may not express any functional recombina-  
5 recombina-  
cells may be unchanged.

**Example 3.** Construction of AdLC8 and AdLC8c Helper Viruses.

The plasmids were constructed according to  
10 standard protocols (Sambrook et al. 1989). The AdLC8 (Fig. 11A) helper virus was rescued by cotransfection of 293 cells with pLC8 having a "floxed" packaging signal ( $\Psi$ ), and pBHG10. pLC8 was constructed as follows. A 9.6 kb *AscI* fragment was removed from pBG18 (Bett and  
15 Graham unpublished) a plasmid containing the majority of the adenovirus genome, including the Ad packaging signal and ITRs, generating pLC4. A synthetic *loxP* site with *Bam*HI compatible ends was obtained by annealing two single-stranded oligonucleotides: 5'-GAT CCA ATA ACT TCG  
20 TAT AGC ATA CAT TAT ACG AAG TTA TAA GTA CTG AAT TCG-3' and 5'-GAT CCG AAT TCA GTA CTT ATA ACT TCG TAT AAT GTA TGC TAT ACG AAG TTA TTG-3'. The *loxP* site was cloned into the unique *Bam*HI site of pLC4, located at nt 188 from the left end of the Ad5 genome, generating pLC5.  
25 This region has been shown previously to tolerate similar insertions of DNA without affecting virus replication (Bautista et al 1991). A second *loxP* site was inserted into pLC5 by first introducing the *loxP* oligonucleotides into the *Bam*HI site of pABS.9, generating pLC7, and subcloning a 1.4 kb *Xba*I fragment, containing the *loxP* site and a neo bacterial-expression cassette, into the unique *Xba*I site of pLC5. Thus, the resulting plasmid, pLC8, contains two *loxP* sites separated by 1452 bp and flanking the adenovirus  
30 packaging signal and neo cassette. The AdLC8luc helper virus is similar to AdLC8, but lacks the neo expression

cassette in E1, and contains the firefly luciferase gene under the regulation of the human cytomegalovirus (HCMV) major immediate-early promoter (-299 to +72 bp relative to the transcription start site) and simian virus 40 polyadenylation signal (pA) inserted into the E3-deleted region. AdLC8cluc was rescued by cotransfection of 293 cells with pLC8c and pUMA7L. pLC8c is a derivative of pLC8 from which the neo cassette was removed by digestion with *Swa*I followed by recircularization.

pUMA7L is a derivative of pBHG10 that contains firefly luciferase gene (DeWet et al. 1987) under the regulation of the HCMV immediate-early promoter and SV40 pA. The 2.2 kb *Bgl*III fragment, containing the HCMV-luciferase expression cassette was removed from pCA18 (Addison and Graham, unpublished) and ligated into the unique *Bam*HI site of pABS.10 (Bett 1995), generating pABS10lucr. pABS.10lucr was then partially digested with *Pvu*I in order to linearize the plasmid, and inserted into the *Pac*I site of pBHG10, generating pUMA7L.

**Example 4.** Construction of a Helper-Dependent Vector. pCA35, which contains the *E. coli*  $\beta$ -galactosidase (*lacZ*) open reading frame under the control of the murine cytomegalovirus (MCMV) immediate-early promoter, was digested with *Sal*I, repaired with the Klenow fragment of DNA polymerase, and recircularized, generating pCA35KS. The MCMV-*lacZ* expression cassette was excised from pCA35KS by digestion with *Xba*I/*Bam*HI and inserted into *Xba*I/*Bam*HI digested pABS.4, generating pABS.4MClacZ. This plasmid was then linearized with *Sal*I and ligated into the unique *Xho*I site of pFG140dx3, a derivative of pFG140 (Graham 1984) that is deleted for sequences between 16.1 and 83.6 map units of the Ad5 genome, generating pUMA10R. A 1276 kb *Swa*I fragment from pUMA10R was removed and replaced by a 8270 bp *Sma*I fragment from

bacteriophage lambda DNA (31617 to 39888 bp of the conventional lambda map), generating pRP1001.

**Example 5. Efficiency of Excision of Packaging Signals from AdLC8.**

5           The kinetics and efficiency of excision by the Cre recombinase in the 293Cre1 cell line, and the ability of  $\Psi$  viral DNA to replicate. 293Cre1 cells, and 293 cells as controls, were infected with AdLC8 at an MOI of 10 PFU per cell. DNA was purified at various  
10 times post-infection, digested with PvuI, separated on a 0.8% agarose gel and the products examined by ethidium bromide staining. Fig. 11B demonstrates that excision of the loxP flanked segment is almost complete as evidenced by complete elimination in 293 Cre1 cells of  
15 the 1.1 kb band and almost complete disappearance of the 3.5 kb band both constituting the left end of the parental virus AdLC8. Instead, a 3.2 kb band is detectable from 12 hr onwards, which represents the viral band generated by excision of the floxed cassette.  
20 The excision product of 1.5 kb is not detectable on the agarose gel since it remains unreplicated due to a lack of an origin for DNA synthesis. Furthermore, Fig. 11B shows that the recombined virus (AdLC8 $\Psi$ ) in the 293Cre1 line, replicates as well as the parental AdLC8 virus on  
25 293 cells.

Southern blot analysis was performed to further examine the kinetics of Cre-mediated recombination. The agarose gel shown in Fig. 11B was transferred to a nylon membrane and probed with a  
30 partially-purified 4.4 kb BstXI-BglII labelled fragment from pLC8. The probe hybridized with fragments of 3489 and 1123 bp of AdLC8, and with a 1452 bp (representing the excised circle) and a 3160 bp fragment of the recombined virus (Fig. 11C). Furthermore, this probe  
35 hybridized with a 9777 bp fragment representing the right end of both viruses. Additional bands of

approximately 4.6, 2.2, and 1.7 kb were detected due to contamination of the probe with the 5.3 kb BstXI fragment of pLC8. Over-exposure of membrane during autoradiography revealed that no recombination products were detectable prior to 6 hr post-infection in the 293Cre1 line. This suggest that the extreme left end of the virus may not be accessible by Cre during early times after infection, even though Cre is produced constitutively by 293Cre1 cell line and is present at the time of infection. Analysis of later time point using a shorter exposure time demonstrated that by 12 hr post-infection approximately 80% of the viral DNA had undergone recombination, and this fraction did not increase at later time points. The excised circle represented by a 1.5 kb band is detectable from 9 hr onward by the more sensitive Southern blot technique, and does not appear to be replicated. No recombination products (3.2 kb and 1.5 kb bands) are detectable after infection of 293 cells with AdLC8, indicating that the recombination products observed in the infected 293Cre1 cells derive from Cre-specific events. Since the quantity of unrecombined viral DNA increases with time with kinetics similar to that of the total DNA, we conclude that this represents virus that has entered the cell and is replicated, and is not due to residual virus that has not infected the cell. It is not known at present why these viral genomes escape Cre-mediated recombination, but it may reflect saturation of the Cre protein in the 293 cell line. Nevertheless, this illustrates that the packaging signal is efficiently excised from the majority of the ADLC8 virus in the 293Cre1 cell line, and removal of this sequence does not affect viral DNA replication.

**Example 6.** Amplification of AdRP1001 by AdLC8 and AdLC8cluc.

Semiconfluent monolayers of 293 cells (60 mm) were transfected by calcium phosphate precipitation (Graham and van der Eb 1973) with 5 $\mu$ g of pRP1001 for 4 hr at 37°C, the transfected cells were infected at a multiplicity of infection (MOI) of 10 with AdLC8 or AdLC8cluc, the medium replaced, and the cells incubated until the monolayers showed complete CPE (48-72 hours). The cells were scraped into the medium and the virus released by 3 rounds of freezing and thawing. An aliquot of the resulting crude viral lysate (500  $\mu$ l) was serially passaged on 60 mm dishes of 293Cre1 or 293Cre4 cells. During each round of amplification of the helper-dependent vector, the 293Cre cells were coinfecting with AdLC8 or ADLC8cluc at an MOI of 5 for the first 2 rounds of amplification and at MOI of 1 for subsequent passages. Amplification of AdRP1001 was monitored by assaying an aliquot of the crude viral lysate after each passage for the presence of lacZ-expressing virus (blue forming units, BFU) on 293 cells. 293 monolayers were infected for 30 minutes with the virus inoculum (500 $\mu$ l), and the infected monolayers incubated for 24 hours at 37°C. The infected monolayers were washed once the PBS<sup>2+</sup>, fixed with 0.5 ml of 0.2% glutaraldehyde, 2% para-formaldehyde, 2mM MgCl<sub>2</sub> for 5 minutes at 37°C, washed, and stained with X-gal (5mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 5mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 2mM MgCl<sub>2</sub>, 1 mg/ml 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside [X-gal] in PBS). Plates were incubated overnight at room temperature, and the number of BFU determined by visual inspection. Viral lysates were also monitored for the presence of helper virus and RCA by plaque assays on 293 lysates were also monitored for the presence of helper virus and RCA by plaque assays on 293 and A549 monolayers, respectively. The frequency of AdLC8-mediated conversion of PRP1001 into packagable linear genomes was about 800 BFU/ $\mu$ g transfected DNA.

293Cre1 cells were transfected with pRP1001, a plasmid that is deleted for most of the adenovirus sequences (Fig. 12A) and then infecting the transfected cells with the AdLC8 helper virus 18 hours later.

- 5 Serial passage of AdRP1001 through AdLC8-infected 293Cre1 cells resulted in an increase in the titer of helper-dependent vector at each passage (Fig. 12B). After five serial passages, the titer of AdRP1001 was approximately  $10^8$  BFU/ml. The quantity of AdLC8 that had
- 10 escaped the excision of packaging signals and the formation of RCA was monitored by plaque assays on 293 and A549 cell lines respectively. The level of AdLC8 present after each passage remained relatively constant at  $5 \times 10^5$  PFU/ml (Fig. 12B), and was comparable to the
- 15 relative amount of unrecombined AdLC8 DNA observed during Southern blot analysis (Fig. 11C). RCA were detected after the second amplification through 293Cre1, and RCA titers continued to increase with subsequent passages. That this was indeed RCA was determined by
- 20 analysis of the DNA content of several plaque isolates. Restriction enzyme analysis of DNA from cesium chloride-purified virus also showed that the final viral preparation contained restriction patterns consistent with the presence of 3 distinct viral populations:
- 25 AdRP1001, AdLC8, and E3-deleted RCA.

- Using the same amplification strategy outlined for AdLC8, the AdRP1001 vector was amplified to high titers with AdLC8cluc. (Fig. 13A) The frequency of AdLC8cluc-mediated conversion of pRP1001 into packagable
- 30 linear genomes was similar to that of AdLC8 (about 1400 BFU/ $\mu$ g of transfected DNA in AdLC8cluc-infected 293 cells). Serial passage of AdRP1001 through AdLC8cluc-infected 234Cre1 cells resulted in an approximately 10-fold increase in the titer of AdRP1001 per passage.
- 35 Similar kinetics of amplification were observed for other vectors ranging in size from 27.7 kb to 33.6 kb.



Furthermore, the quantity of AdLC8cluc present in stocks remained low, and during the seventh passage, constituted less than 1% of the total virus. Finally, no RCA were detected. The data presented in Fig. 13B clearly illustrates that AdLC8cluc may be employed as a helper virus to produce high titer stocks of AdRP1001.

**Example 7. Large Scale Virus Preparations of AdRP1001.**

Large scale virus preparations of AdRP1001 were performed by infecting 150 mm dishes of 293Cre with 1 ml of crude AdRP1001 stock per 150 mm dish (MOI = ~1) supplemented with  $2 \times 10^7$  PFU of helper virus. After complete CPE, purification of AdRP1001 virions was performed by CsCl buoyant density centrifugation as previously described. After centrifugation, fractions were collected through the viral bands, and assayed for the presence of luciferase-expressing virus. Each fraction was diluted 1:1000, and an aliquot (500 $\mu$ l) was used to infect 293 monolayers. Twenty-four hours post-infection, crude protein extracts were prepared from the infected cells and assayed for luciferase activity using a commercial kit (Promega) and a Berthold model LB9501 luminometer. Luciferase activity ( $R^2 = 0.963$ ) correlated well with the relative titer of AdLC8cluc, indicating that luciferase activity is a good indicator of the level of contaminating helper virus. Using the relationship shown in Fig. 14, 1 PFU AdLC8cluc =  $\sim 2.6 \times 10^5$  pg luciferase per  $10^6$  infected cells.

AdRP1001 and helper virus titers were also isolated from the lower density band after CsCl centrifugation. We obtained a titer of  $8.6 \times 10^9$  BFU/ml, or a total yield of  $6.5 \times 10^9$  AdRP1001 virions from 20-150 mm dishes of 293Cre cells. The ratio of AdRP1001 to AdLC8cluc in this stock was  $\sim 1:16,000$ , or a 30-fold enhancement in the purity of AdRP1001 over the crude stock used in the purification. This is in contrast to helper-dependent systems developed previously in which

the major component of the "purified" vector was helper virus, with between 100 to 1000 fold more helper virus than vector. (Mitani et al. 1995, Fisher et al. 1996). In addition, our yield of nearly  $10^{10}$  virions is ~1000  
5 fold higher than that obtained using these other systems. Furthermore, it has been shown that the vectors in these systems underwent DNA recombination at high frequency, generating a heterogeneous population of vector consisting of monomer, dimer and more complex  
10 DNA structures. In contrast, restriction analysis of purified AdRP1001 showed that the DNA structure of the vector was identical to passage vector and restriction maps predicted from the sequence of the initial  
transfecting plasmid pRP1001. (Fig. 4B) We conclude  
15 that the helper-dependent system described above is capable of producing high titer vector preparations that contain very low quantities of helper virus.

CLAIMS

We claim:

1. A recombinant adenovirus vector system for expressing foreign genes, comprising:
  - 5 (a) a first adenovirus vector having a modified early region 1 (E1) in which the packaging signals of the virus are flanked on either side by target sites that are recognized by a recombinase, and
  - (b) a cell line that is able to support replication of the virus of 1. (a) and which additionally expresses the recombinase Cre that is able to catalyze site specific recombination between *lox P* sites, and
  - 10 (c) a second vector, having
    - (i) a deletion of large portions of the viral genome, up to approximately 35,000 bp but retaining Ad viral sequences required in cis for viral DNA replication and packaging of viral nucleic acid into virions, namely the left and right ITRs and the packaging signal and
    - 15 (ii) a fragment or fragments of foreign nucleic acid of about 35,000 bp in size.
2. A recombinant adenovirus vector system according to claim 1, wherein the recombinase is Cre and the target sites are *loxP*.
- 25 3. A plasmid used for making a vector (helper virus) according to claim 1(a), said plasmid comprising a modified circular adenovirus genome wherein sequences from region E3 of the viral genome are replaced by (a) the packaging signal from the viral genome bracketed by target sites for a recombinase, (b) a bacterial plasmid origin of replication and (c) an antibiotic resistance gene.
- 30

4. A plasmid according to claim 3, wherein the recombinase is Cre, the target sites are *loxP* and the antibiotic is ampicillin.

5. A plasmid used for making a vector (helper-dependent virus) according to claim 1(c), comprising the left and right ITRs and packaging signal of the viral genome, a plasmid origin of replication and an antibiotic resistance gene, and a foreign nucleic acid of up to 35,000 bp in length.

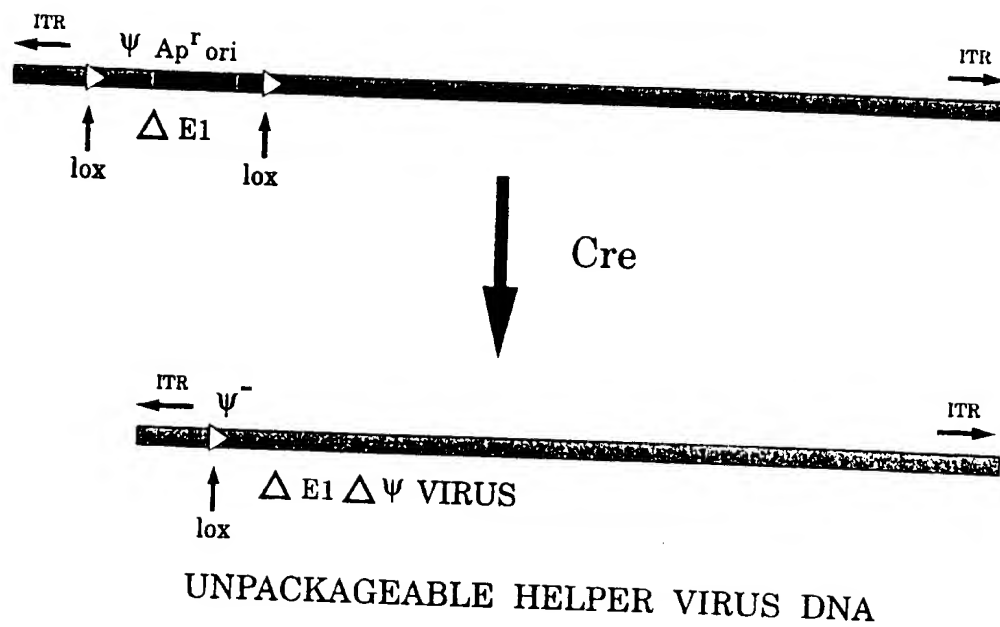
10 6. A plasmid according to claim 5, wherein the antibiotic is ampicillin.

7. A method of producing a helper virus genome that is incapable of packaging its nucleic acid into virions retains viral functions that can act in trans to complement the replication of a second, helper dependent vector from which all or most of the viral genes have been deleted and substituted with a foreign nucleic acid, comprising:  
coinfecting a cell line that supports replication of an adenovirus vector with (a) a first vector having a modified early region 1 (E1) such that the packaging signals of the virus are flanked on either side by *loxP* sites that are recognized by the Cre recombinase, and (c) a second vector, having  
25 (i) a deletion of large portions of the viral genome, up to approximately 35,000 bp but retaining Ad viral sequences required in cis for viral DNA replication and packaging of viral nucleic acid into virions, namely the left and right ITRs and  
30 the packaging signal, and  
such that the *loxP* sites flanking the packaging signal of the first vector will be acted upon by the Cre

35

recombinase expressed in said cells to induce excision  
of said packaging signal.

1/16



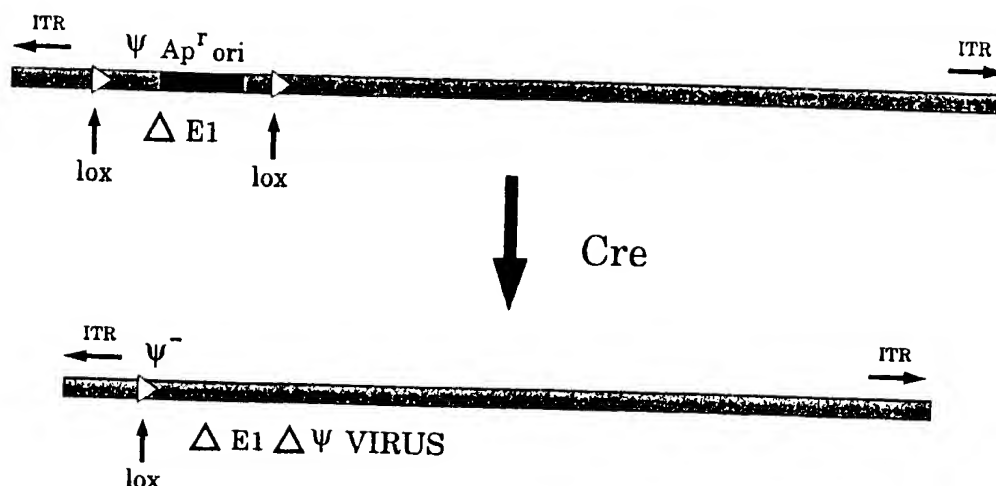
+



FIG. 1

SUBSTITUTE SHEET (RULE 26)

2 / 16



UNPACKAGEABLE HELPER VIRUS DNA

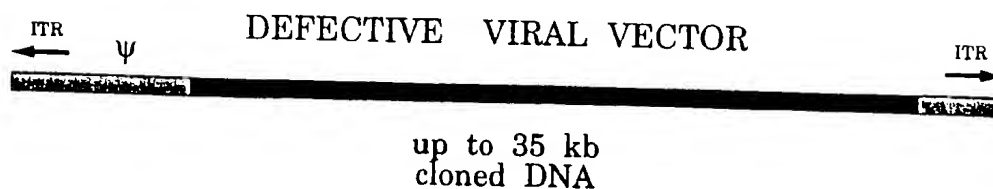
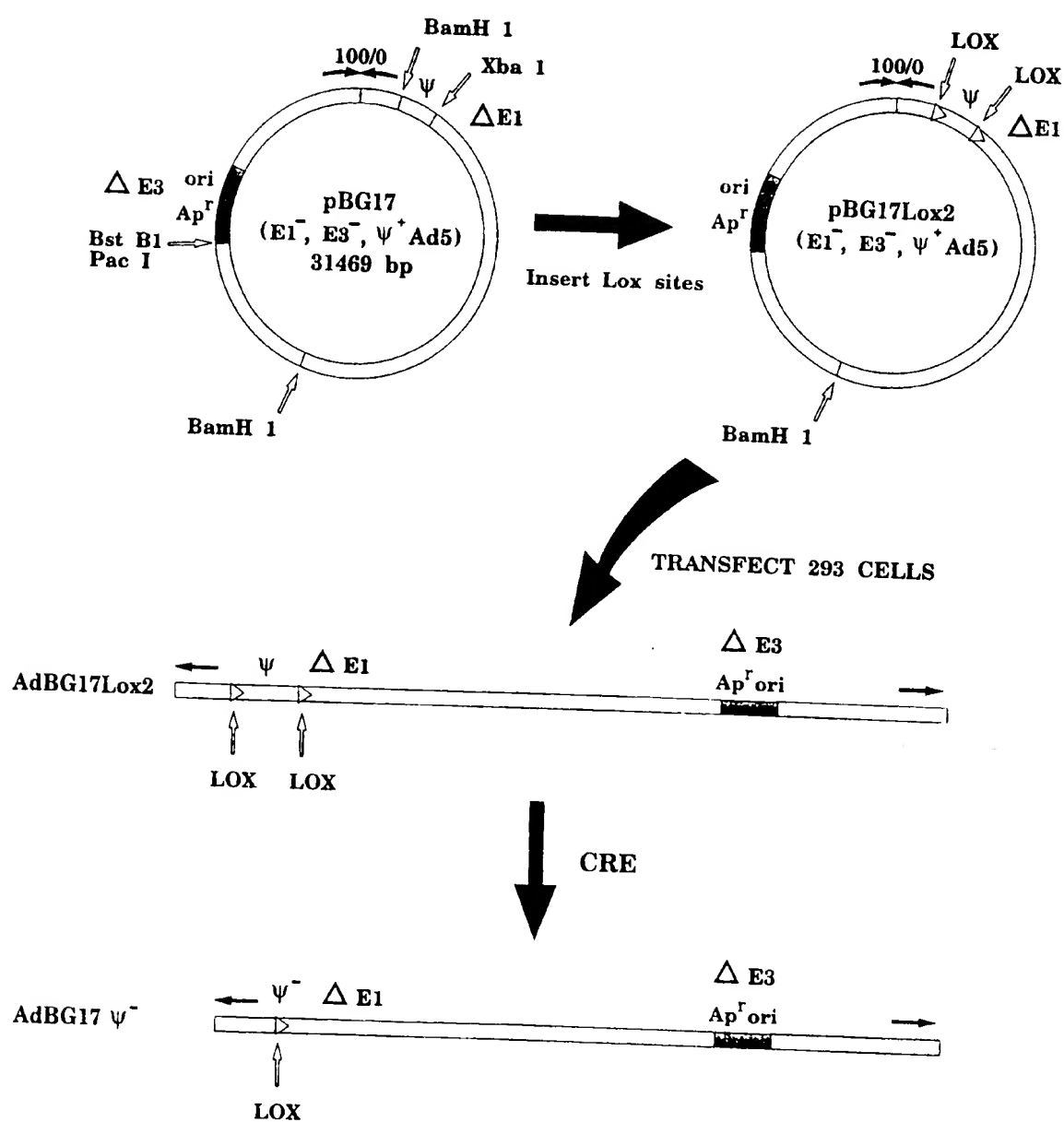
PROVIDES ESSENTIAL VIRAL FUNCTION  
IN TRANS (HELPER FUNCTIONS) FOR:REPLICATION OF BOTH VECTOR & HELPER DNA:  
PACKAGING OF VECTOR DNA ONLY

FIG. 2

SUBSTITUTE SHEET (RULE 26)

3/16



**FIG. 3**  
SUBSTITUTE SHEET (RULE 26)



4/16

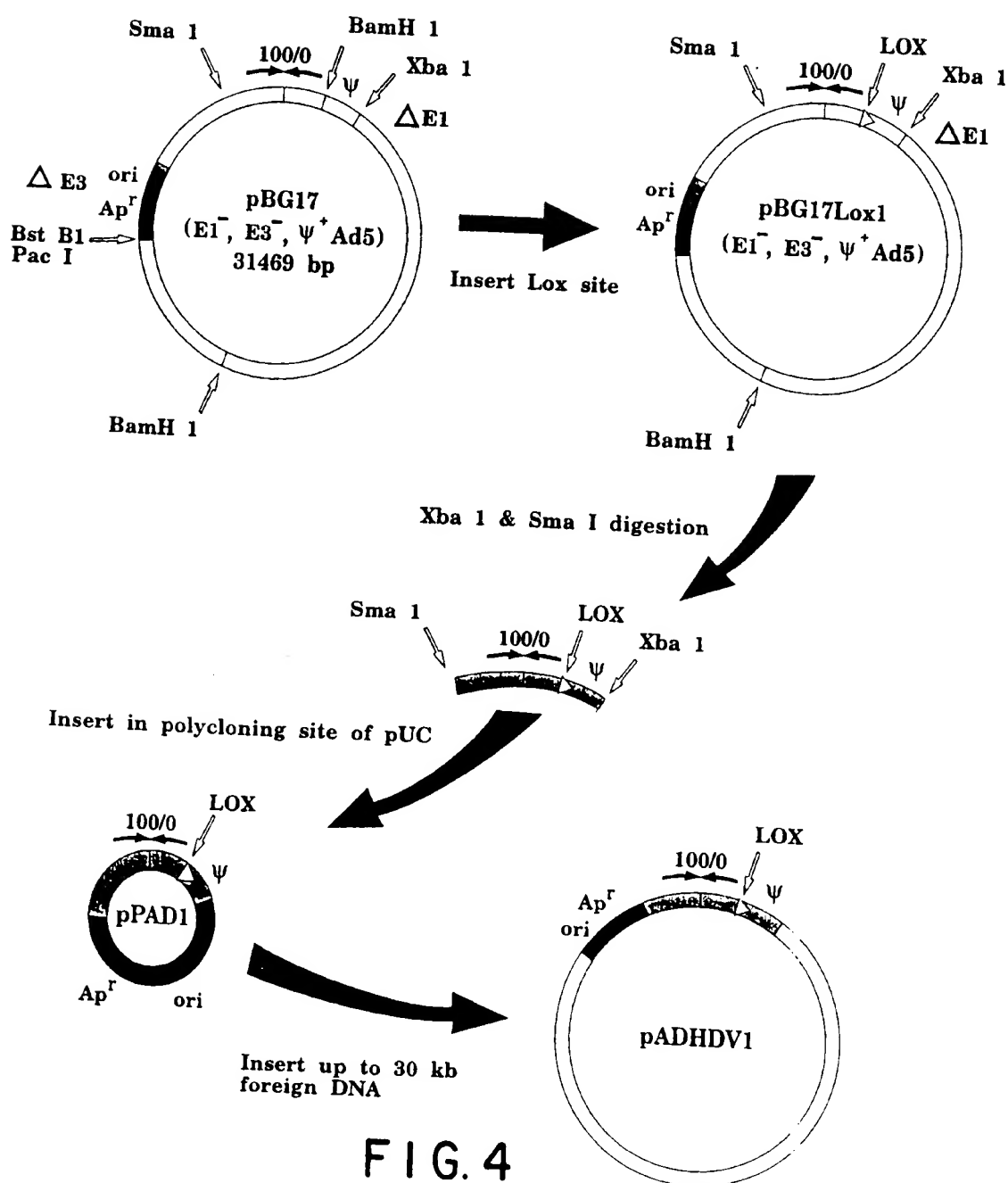


FIG. 4

5/16

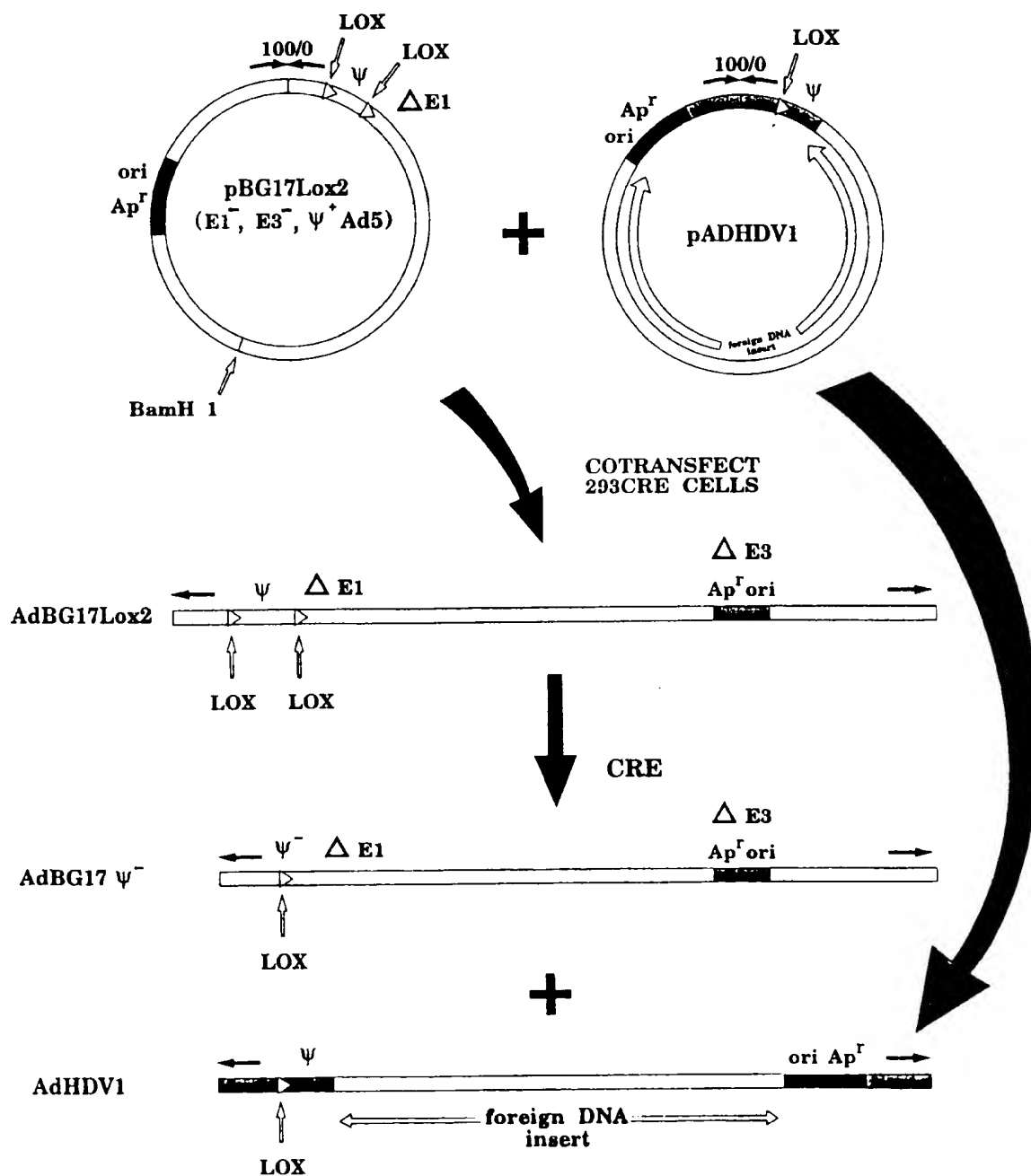


FIG. 5

6/16

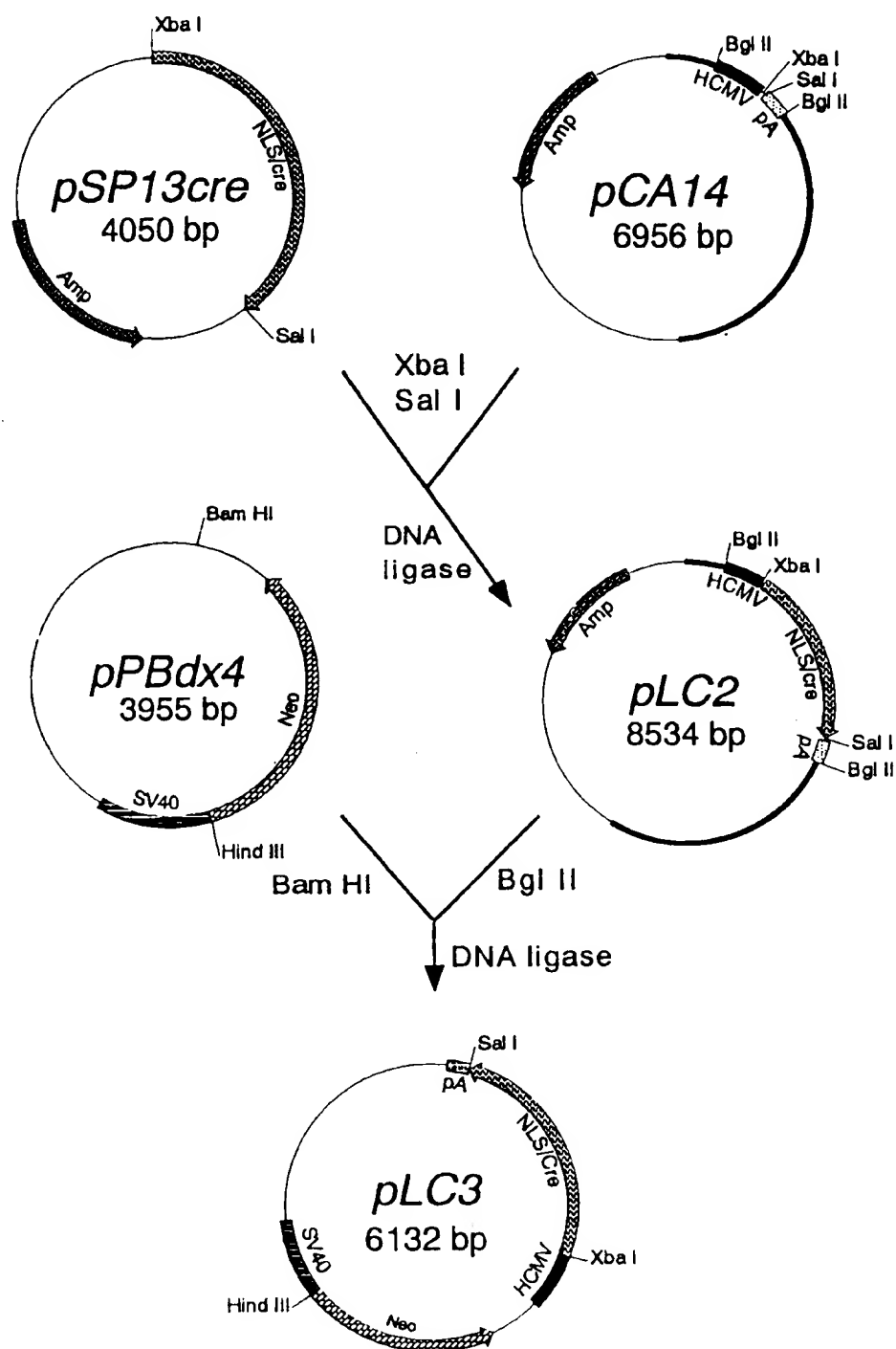


FIG. 6

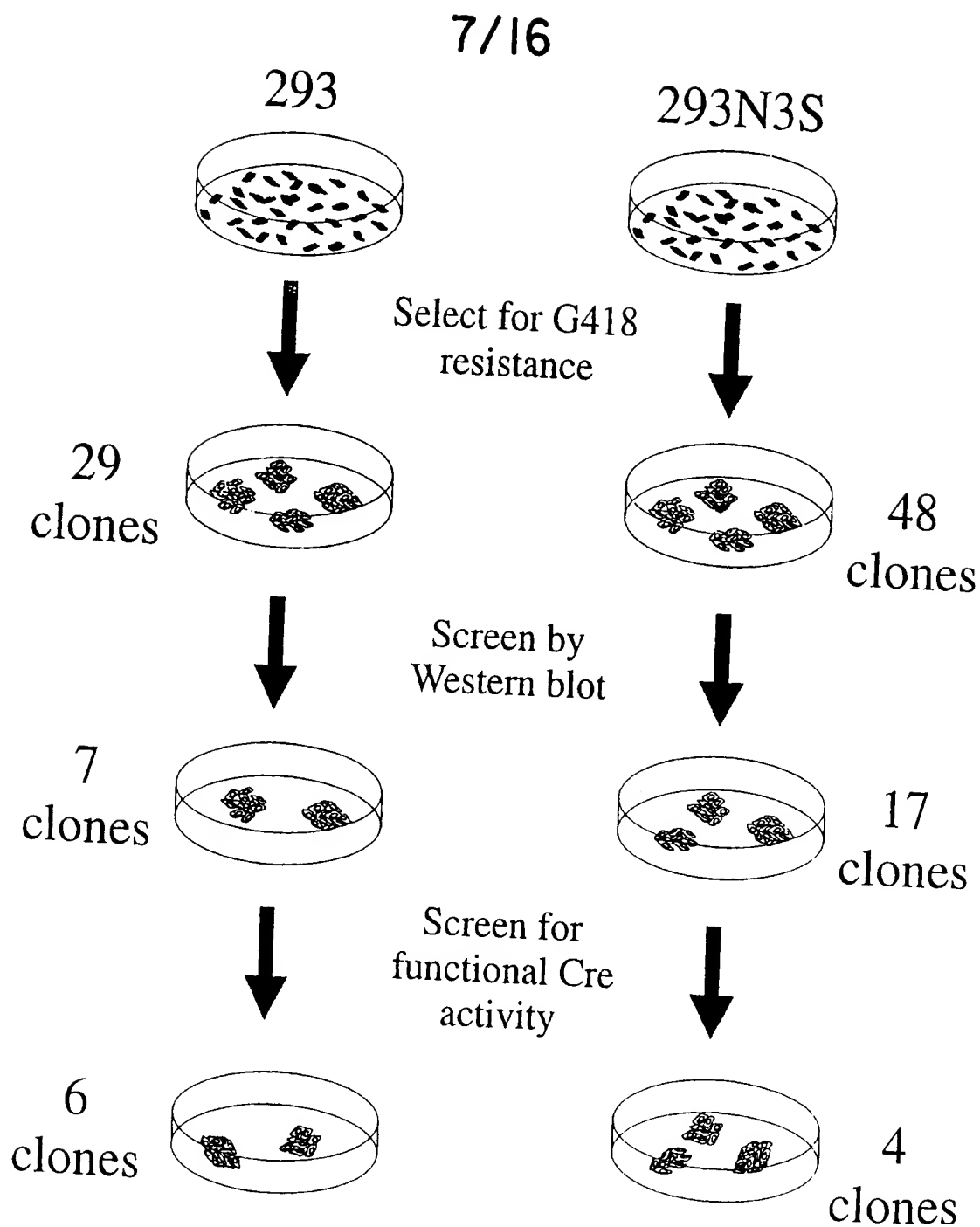
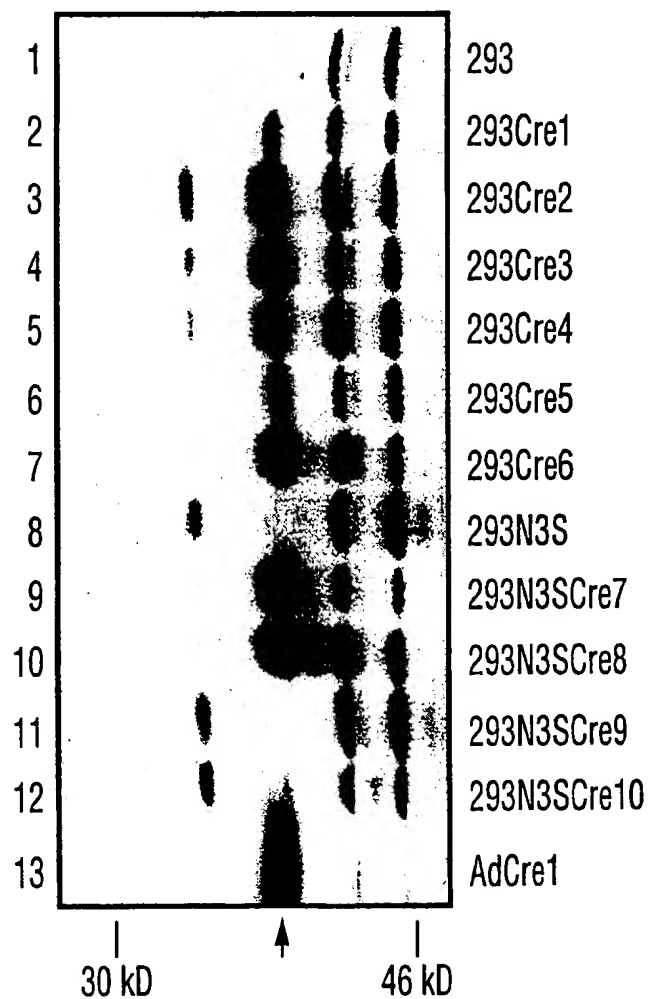


FIG. 7

SUBSTITUTE SHEET (RULE 26)

8/16

**FIG. 8**

SUBSTITUTE SHEET (RULE 26)

9/16

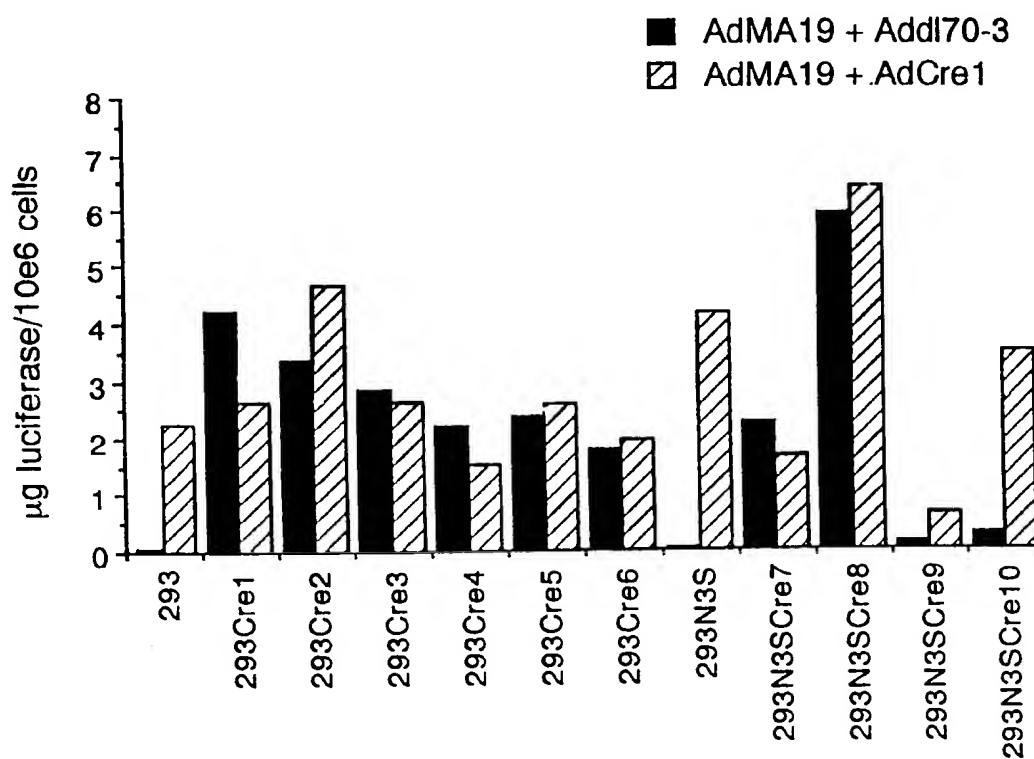


FIG. 9

SUBSTITUTE SHEET (RULE 26)

10/16

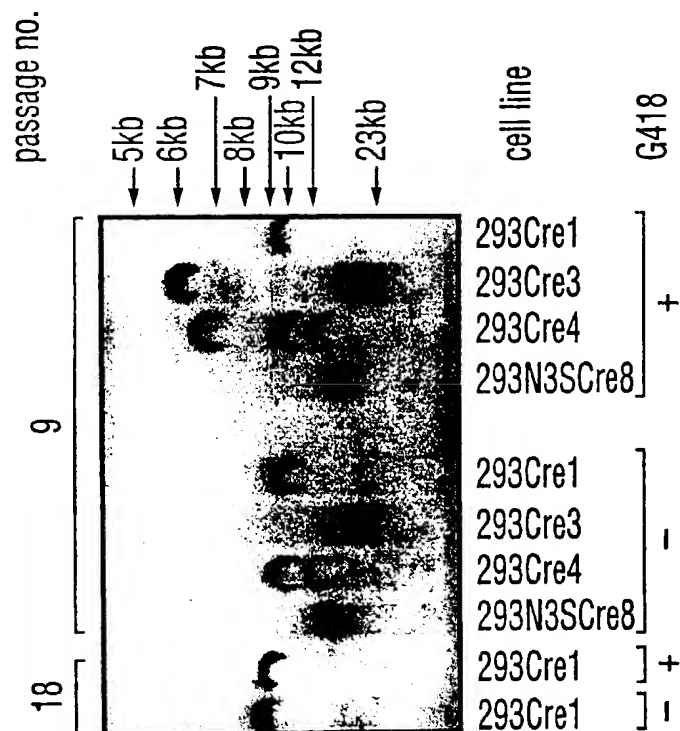


FIG. 10A

11/16

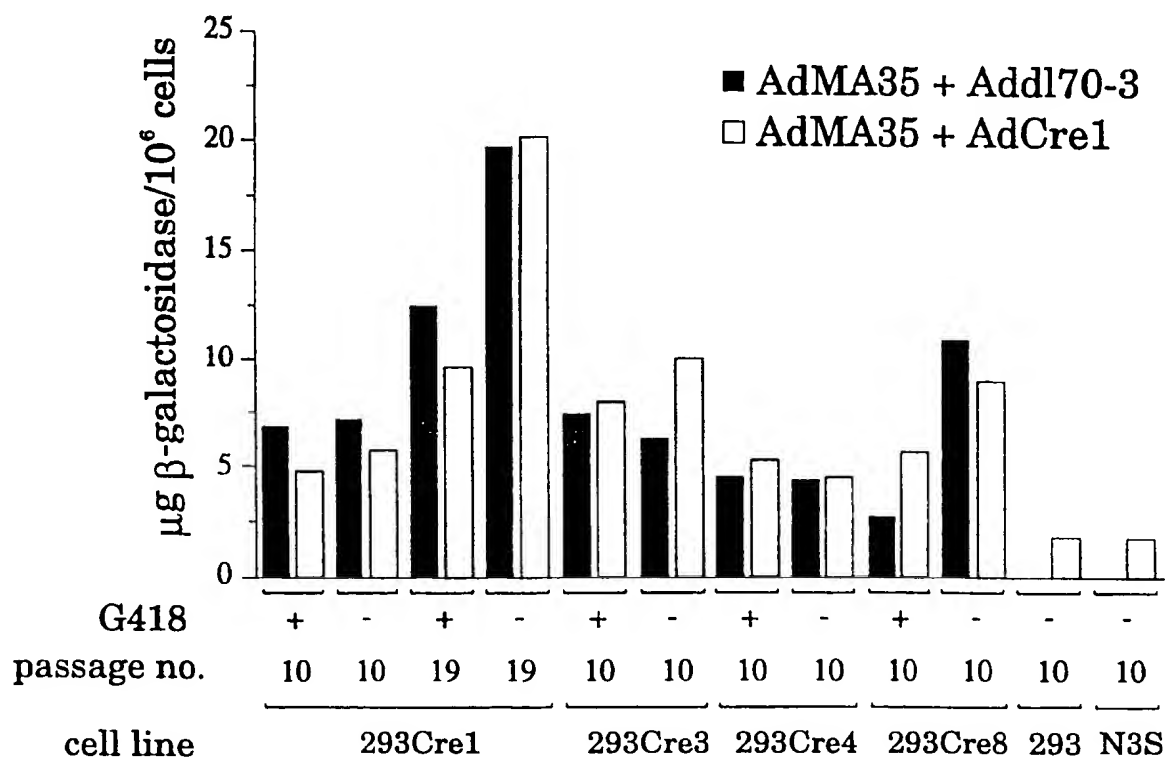


FIG. 10B



12/16

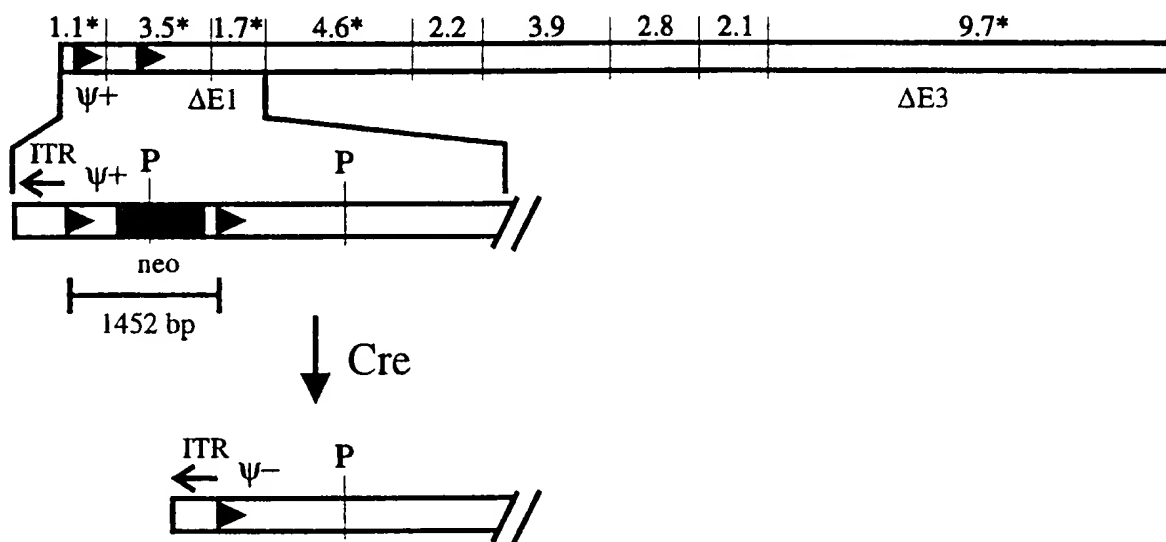


FIG. IIA

SUBSTITUTE SHEET (RULE 26)

13/16

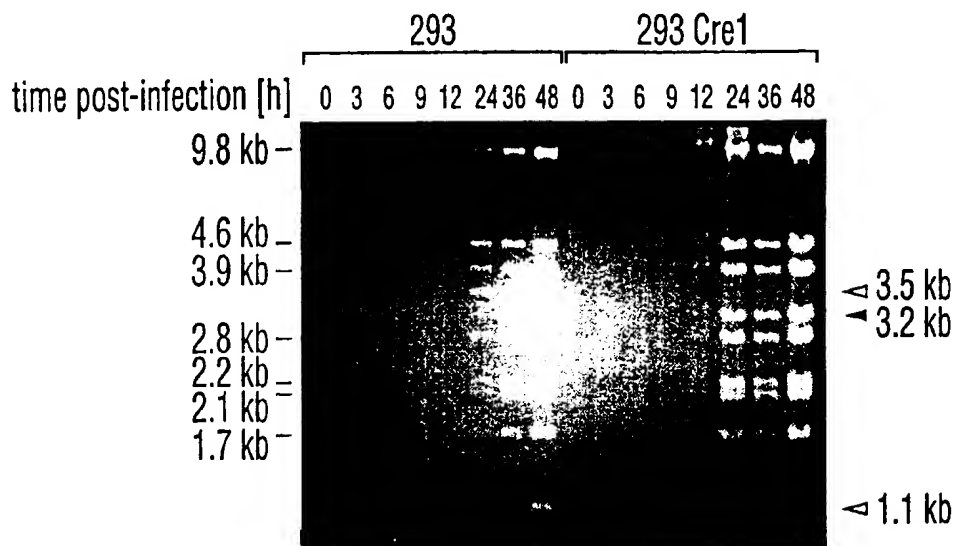


FIG. 11B

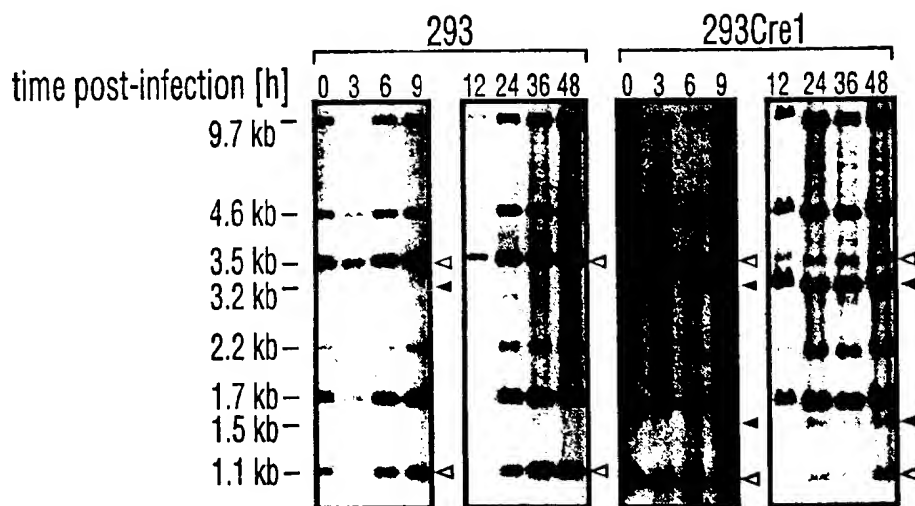


FIG. 11C

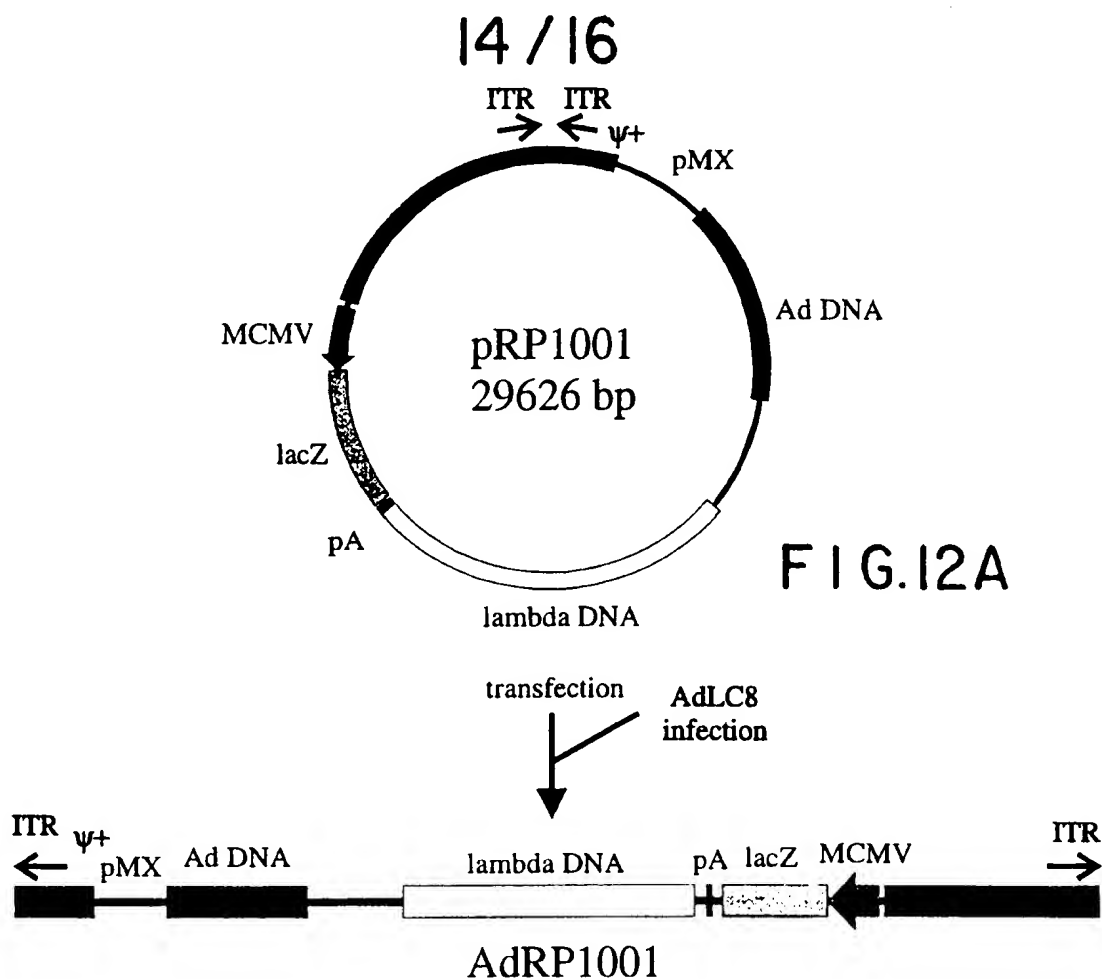
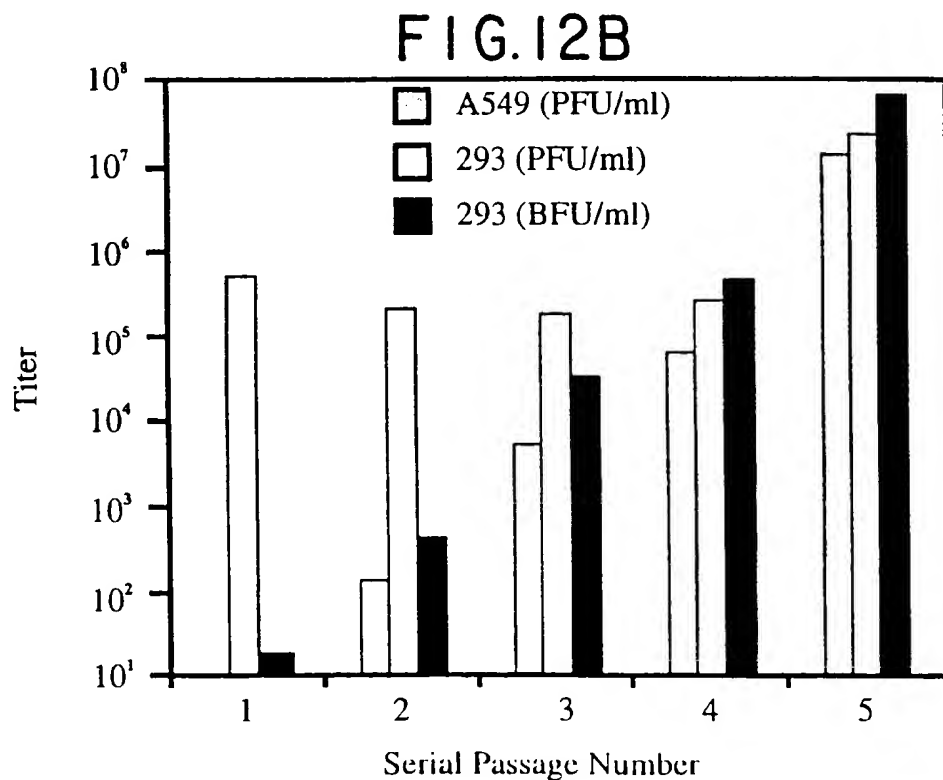


FIG.12A



15/16

FIG. 13A

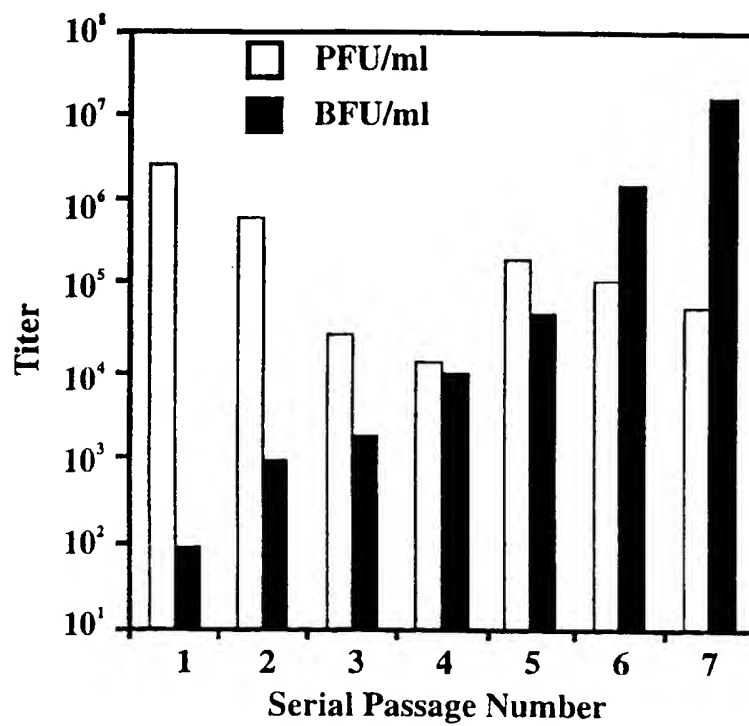
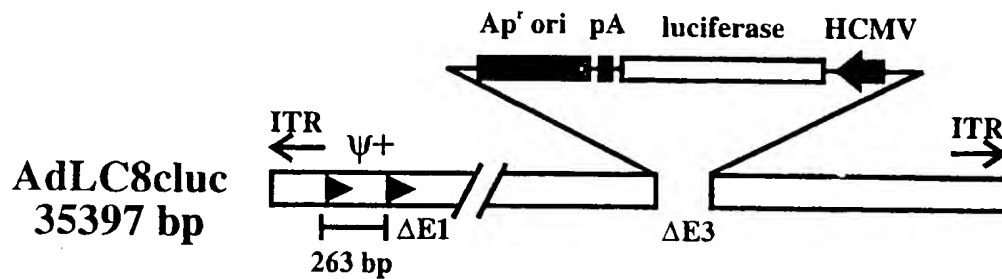


FIG. 13B

16/16

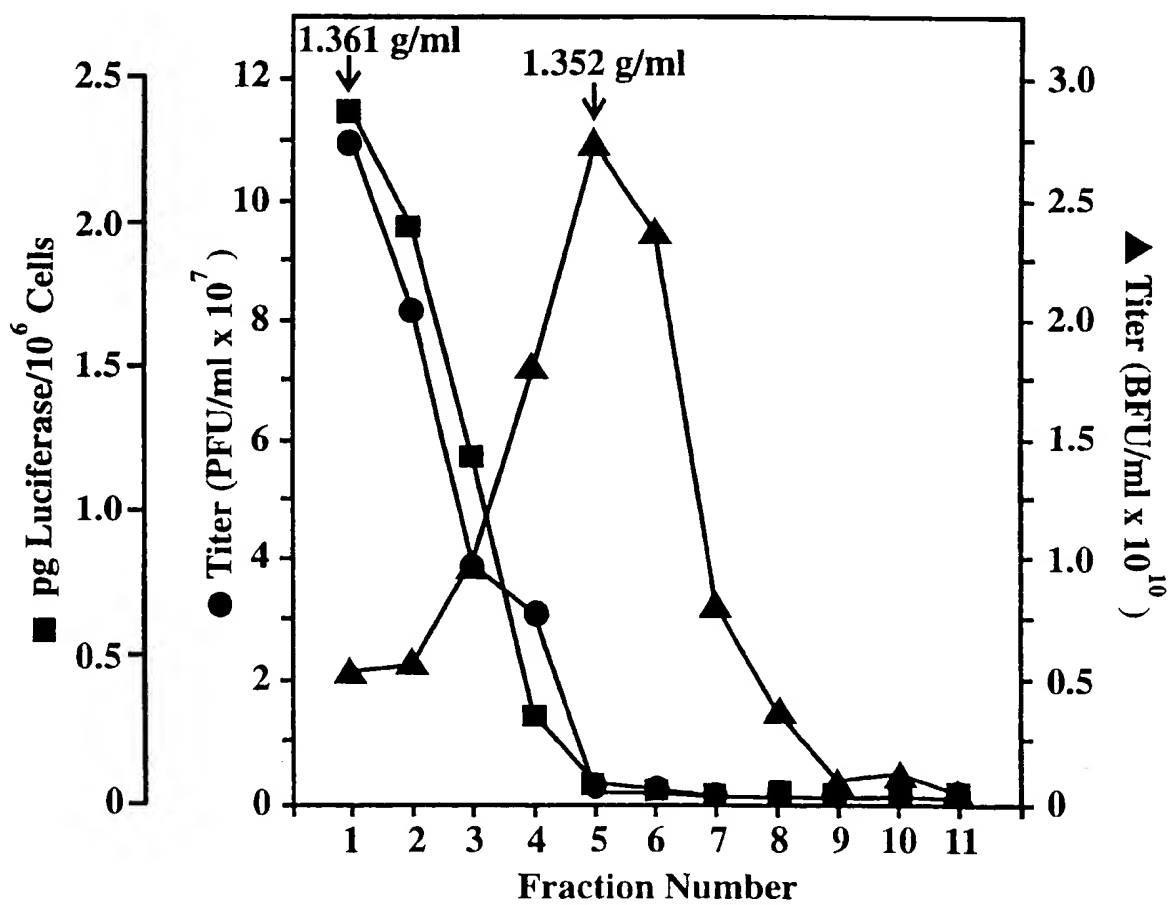


FIG. 14

## INTERNATIONAL SEARCH REPORT

International Application No

PCI/CA 96/00375

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/86

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROC. NATL.ACAD SCI., vol. 91, September 1994, NATL. ACAD SCI., WASHINGTON, DC, US;; pages 8802-8806, XP002011773 A.J. BETT ET AL.: "An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3" cited in the application see the whole document ---	1-7
A	WO,A,95 00655 (MC MASTER UNIVERSITY) 5 January 1995 cited in the application see the whole document --- -/--	1-7



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*&\* document member of the same patent family

Date of the actual completion of the international search

27 August 1996

Date of mailing of the international search report

03.09.96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax (+31-70) 340-3016

Authorized officer

Hornig, H

## INTERNATIONAL SEARCH REPORT

International Application No  
PC/CA 96/00375

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	EP,A,0 704 534 (SUMITOMO PHARMA) 3 April 1996 see the whole document ---	1-7
P,A	NUCLEIC ACIDS RESEARCH, vol. 23, no. 19, 11 October 1995, IRL PRESS LIMITED, OXFORD, ENGLAND, pages 3816-3821, XP002011774 Y. KANEGAE ET AL.: "Efficient gene activation in mammalian cells by using recombinant adenovirus expressing site-specific Cre recombinase" see the whole document ---	1-7
P,A	J. VIROLOGY, vol. 69, no. 8, August 1995, AM.SOC.MICROBIOL., WASHINGTON, US, pages 4600-4606, XP002011775 M. ANTON AND F.L. GRAHAM: "Site-specific recombination mediated by an adenovirus vector expressing the Cre recombinase protein: a molecular switch for control of gene expression" cited in the application see the whole document ---	1-7
P,X	WO,A,95 27071 (UNIV TEXAS) 12 October 1995 see page 10, line 27 - line 36 see page 21, line 23 - page 23, line 24; claims 14,15 -----	5

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 96/00375

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9500655	05-01-95	AU-B- 7118494	17-01-95
		CA-A- 2166118	05-01-95
		EP-A- 0705344	10-04-96
-----			
EP-A-0704534	03-04-96	JP-A- 8084589	02-04-96
		AU-B- 3024895	04-04-96
		CA-A- 2157063	20-03-96
-----			
WO-A-9527071	12-10-95	AU-B- 2238695	23-10-95
-----			